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Porcine reproductive and respiratory syndrome virus: understanding and managing persistent infection

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Dedication

This thesis is dedicated to my family, who offered me unconditional love and support throughout the course of my studies.

To my wife Maria Esthela, who has been a great source of motivation and has supported my desire to further my education despite the sacrifices implied. I love you.

To my children: Jose Roberto, Mayra Alejandra and Luis Fernando, who are the reason to try and make our lives better. I thank for enabled me to complete this work and tolerate my absences.

To my parents, Jose Ramon Molina G, and Dolores Barrios A., who taught me the value of education and who made sacrifices for us, their children, so that we could have the opportunities they did not have. (A mis padres, José Ramón Molina y Dolores Barrios Ángeles, quienes me enseñaron el valor de la educación e hicieron sacrificios para que nosotros, sus hijos pudiéramos tener las oportunidades que ellos no tuvieron)

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Abstract

This experiment was designed as a longitudinal study in which pigs were followed for up to 202 days post inoculation (DPI). On day 0, 109 3-week-old pigs were intramuscularly inoculated with PRRSV strain VR-2332. Negative control pigs (n = 56) were sham inoculated with MEM by the intramuscular route. Thereafter, at approximately 2-week intervals, samples were collected from all animals and a subset of randomly selected animals was euthanized and tissues collected. The presence and amount of virus was assessed using qRT-PCR, standard virus isolation, and bioassay. Detection of PRRSV in serum by qRT-PCR showed that most pigs cleared the viremia by 42 DPI, but some pigs continued to test positive up to 154 DPI. Lymphoid tissue was qRT-PCR positive through 202 DPI in one or more pigs at each sampling point. Infectious virus was recovered from serum and lymphoid tissue by virus isolation on MARC-145 cell culture in a few pigs up to 28 DPI. Swine bioassays based on lymphoid tissue homogenate showed that infectious virus was present in these tissues up to 175 DPI. These results suggest that infectious virus is able to persist in populations for a longer period of time than previously thought. RT-PCR was the most sensitive assay for detecting PRRSV, but the discrepancy between PCR and bioassay results indicated that PCR is detecting non-infectious virus.

A subset of 89 PRRSV-inoculated pigs (donor pigs) and 46 negative control pigs were selected to estimate the risk of PRRSV transmission via ingestion of muscle. Beginning on DPI 28, serum, lymphoid tissues, and muscle (*M. longissimus dorsi*) samples were collected from euthanized pigs. A total of 7 of 89 (7.7%) serum samples, 62 of 89 (69.6%) lymphoid tissues samples, and 13 of 89 (14.6%) muscle samples were positive by qRT-PCR. Swine transmissibility studies were performed by feeding thirteen 3-week-old PRRSV-naive pigs (recipient pigs) qRT-PCR-positive muscle, and monitored by qRT-PCR for evidence of PRRSV viremia. No transmission of PRRSV to recipient pigs via consumption of muscle samples was observed.

To explore possible prognostic combinations of cell-mediated and humoral immune responses, 3-week-old pigs (n = 10) were intramuscularly (IM) inoculated with PRRSV isolate VR-2332 and followed for 193 days post inoculation (DPI). Negative control pigs (n = 10) were IM inoculated with minimum essential medium (MEM). At ~2-week intervals,

blood samples were collected from all animals and tested for the number of interferon (IFN)-γ-secreting peripheral blood mononuclear cells (Elispot), PRRSV viremia (qRT-PCR), and serum antibodies using PRRSV protein ELISAs (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) and a commercial PRRSV ELISA (IDEXX Laboratories, Inc.). All pigs were viremic by 7 days post inoculation (DPI), with 50% of the pigs resolving viremia by 56 DPI. A PRRSV-specific IFN-γ response was detected at DPI 28, reached a plateau at 42 DPI, declined slightly, and remained relatively stable from 56 to 193 DPI. On the basis of ROC area under the curve (AUC) analysis, the ELISAs that most reliably differentiated PRRSV-inoculated pigs from negative control pigs were the commercial ELISA (AUC = 0.97), the N ELISA (AUC = 0.96), and the M 3' ELISA (AUC = 0.93). Multivariate analyses were performed to evaluate the relationship between the immune response and the duration and level of viremia. With all antibody assays and Elispot included in the models, the analysis determined that the serum-virus neutralizing antibody response was the best predictor of both level and duration of viremia. It may be concluded that humoral antibody responses, particularly the commercial ELISA, N ELISA, and M 3' ELISA are the good predictors of prior exposure to PRRSV, but provide little information regarding the ontogeny of the protective immune response. Likewise, cell-mediated immunity based on the number of IFN-γ-secreting lymphocytes appears to be a poor prognosticator of PRRSV infection status.

In addition, three assays were evaluated for their ability to detect antibodies against *porcine reproductive and respiratory syndrome virus* (PRRSV) in porcine muscle transudate ("meat juice") samples. Serum samples were assayed at a dilution of 1:40, and muscle transudate samples were assayed at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40) using a commercial PRRSV antibody enzyme-linked immunosorbent assay (ELISA). Additionally, muscle transudate samples were tested using an indirect fluorescent antibody test (IFAT) at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40). Attempts to assay muscle transudate samples for neutralizing antibodies using a modified fluorescent focus neutralization assay were unsuccessful.

Chapter 1. General Introduction

THESIS ORGANIZATION

This thesis consists of six chapters. Chapter 1 presents a general introduction and review of porcine reproductive and respiratory syndrome virus (PRRSV). Chapter 2 (Viral, immunological, and host correlates of PRRSV persistence and clearance) will be submitted to Veterinary Research for publication. Chapter 3 (Evaluation of the risk of PRRSV transmission via ingestion of muscle from persistently-infected pigs) has been submitted to Transboundary and Emerging Diseases for publication. Chapter 4 (Dynamics of the immune response during acute and chronic stages of the PRRSV infection) has been submitted to Veterinary immunology and Immunopathology, and Chapter 5 (Diagnostic performance of assays for the detection of anti–porcine reproductive and respiratory syndrome virus antibodies in serum and muscle transudate ("meat juice") based on samples collected under experimental conditions) is accepted for publication in the Journal of Veterinary Diagnostic Investigation. For each research paper, references are listed after the discussion section. The last chapter contains the general conclusions of the research studies and suggests possible areas of future research.

Review of the literature: Porcine Reproductive and Respiratory Syndrome Virus

Classification and Organization

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped, positive-sense, single-strand enveloped RNA virus classified in genus Arterivirus, family Arteriviridae, order Nidovirales (Meulenberg et al., 1997). It is a member of the order *Nidovirales*, family *Arteriviridae,* genus Arterivirus (Cavanagh et al., 1997) and is closely related to equine arteritis virus, lactate dehydrogenase-elevating virus of mice and simian hemorrhagic fever virus (Conzelmann et al., 1993; Meulenberg et al., 1997).

The viral genome is approximately 15 kb in size and composed of nine overlapping open reading frames (ORF) designated ORF1a, 1b, 2a, 2b, and 3 through 7 (Meulenberg et

al., 1993; Nelson et al., 1995). ORF1a and 1b comprise more than 75% of the genome and encode the viral RNA-dependent RNA polymerase (Allende et al., 1999; Meulenberg et al., 1993). ORFs 2 to 5 encode four structural glycosylated proteins (GP2, GP3, GP4 and GP5) and the E protein, a small non-glycosylated protein sometimes designated 2b (Dea et al., 2000; Snijder et al., 1999; Wu et al., 2001, 2005). ORF6 encodes a non-glycosylated integral membrane protein (M) and ORF7 encodes the nucleocapside protein (N) (Conzelmann et al., 1993; Meulenberg et al., 1997; Snijder and Meulenberg, 1998). The GP2, GP4, GP5, M, and probably GP3, proteins are associated with the viral envelope (Dea et al., 2000). The major viral envelope protein (GP5) is associated with virus neutralization (Pirzadeh et al., 1998; Gonin et al., 1999), but GP4 contains a minor neutralizing epitope (Bautista et al., 1999; Meulenberg et al., 1997)

Replication

In vivo, fully differentiated primary porcine alveolar macrophages (PAM) constitute the predominant cell target for PRRSV replication (Duan et al., 1997a). PRRSV appears to replicate primarily in activated monocytes, macrophages, and dendritic cells (Halbur et al., 1995; Thacker et al., 1998; Thanawongnuwech et al., 2000). In addition, replication of PRRSV on epithelial germ cells of the seminiferous tubules has been detected by in situ hybridization and immunohistochemistry (Sur et al., 1997). *In vitro*, PRRSV replicates in PAM and in at least three non-porcine continuous cell lines, CL2621, CRL11171, and MA-104-derived MARC-145 cells. Differences in cell permissiveness among the different PRRSV isolates has been reported (Benfield et al., 1992; Kim et al., 1993; Meng et al., 1996).

The entry of PRRSV to the target cells occurs via receptor-mediated endocytosis. The process is mediated by two consecutive steps: the M or M-GP5 protein complex binds to heparan sulfate on the target cell membrane and then the sialic acid on the virus envelope binds to sialoadhesins on target cell membrane (Delputte et al., 2002; Delputte and Nauwynck, 2004; Duan et al., 1997b; Nauwynck et al., 1999; Vanderheijden et al., 2003). The uncoating process for virus entering by receptor-mediated endocytosis occurs in the endosome, where an acidic environment activates fusion of viral membrane to the endosomal

membrane, but direct fusion of PRRSV with endosomal membrane has been reported (Kreutz et al., 1996; Smith et al., 2004). PRRSV E protein is thought to be an ion-channel protein embedded in the viral envelope that facilitates uncoating of the virus and release of the viral genome in the cytoplasm. (Changhee et al., 2006). PRRSV obtains an envelope by budding through the membrane of the smooth endoplasmic reticulum. The mature virion is released from the cell by exocytosis (Dea et al., 2000).

Genetic variability

Based on comparative sequence analysis of the single-stranded RNA viral genome, two genotypic classes of PRRSV are recognized: a European genotype (type 1) and a North American genotype (type 2) (Allende et al, 1999; Meulenberg et al., 1993). Depending on the specific isolates compared and the area of the genome examined, these genotypes share 64-67% nucleotide sequence and 55–80% amino acid sequence homology (Meng et al., 1995; Nelsen et al., 1999). The highest degree of amino acid variability is in ORF5 (GP5) with 89–94% identity among American isolates and 87–99% identity among European isolates (Andreyev et al., 1997; Suarez et al., 1996). In contrast, ORF6 (M protein) has the least amino acid sequence variation (96-100% homology) (Allende et al, 1999; Meng et al., 1995).

Early on, North American genotype isolates were considered more diverse than European genotype isolates. However, more recent evidence suggests that genotype 1 isolates are actually more diverse than genotype 2 isolates. For example, a study of 66 European viruses isolated 1991-2002 showed evidence of on-going genetic drift, with recent isolates possessing increased genetic distance both relative to older isolates and among themselves (Pesch et al., 2005). A Spanish study on PRRSV isolates reported similar conclusions (Mateu et al., 2006). Based on the extensive diversity among genotype 1 isolates in Belarus and Lithuania, a European or Eurasia origin to PRRSV has been postulated (Stadejek et al., 2006).

At a worldwide level, type 1 and type 2 genotypes are co-distributed in areas once assumed to be populated by either one or the other. Type 2 genotype viruses are common in the western hemisphere, but also in East Asian countries, such as Korea, Japan, and China.

Both genotypes have been reported in Thailand (Thanawongnuwech et al., 2004), Austria (Indik et al., 2005) and Hungary (Balka et al., 2008). The pattern of viral genetic diversity may be associated with geographical separation (Cha et al., 2006; Forsberg et al., 2002) and/or commercial relations among countries or regions. (Balka et al., 2008) Adding to the sense of PRRS "viral entropy", it has become apparent that genetically diverse PRRSV strains may exist within a single production system (Batista et al, 2004; Larochelle et al., 2003), on the same farm (Dee et al., 2001; Fang et al., 2007; Kiss et al., 2006), and even within individual pigs (Chang et al., 2002; Dee et al., 2001).

PRRSV genetic diversity could lead to the selection of more virulent viruses and to the emergence or re-emergence of new forms of PRRS (Rowland, 2007). The significant of this issue is highlighted by the fact that biological variability among PRRSV isolates has been demonstrated in terms of the magnitude and duration of viremia, clinical signs, pathological lesions, and the scale of antibody response (Halbur et al., 1995, 1996a; Johnson et al., 2004; van der Linden et al., 2003). Quasispecies evolution has been reported (Rowland et al., 1999) and RNA recombination (Murtaugh et al., 2001; Yuan et al., 1999), but the mechanism(s) driving the evolution of PRRSV has not been elucidated. New outbreaks in herds previously vaccinated with modified live virus (MLV) vaccines suggest that virus evolution may confer the virus with the ability to escape the immune response. On the other hand, an increase in the virulence of PRRSV isolates has been linked to deletions on the non-structural nsp2 gene. The MN184 isolate, an isolate more virulent than the type 2 prototype isolate VR-2332, presents discontinuous deletions in nsp2 (Han et al., 2006). Likewise, PRRSV isolate SY0608 recovered from severe presentations of PRRSV in the mid-eastern region of China also exhibits deletions in the nsp2 gene (Li et al., 2007).

Environmental Stability

PRRSV is fragile and quickly inactivated by heat and drying. At 25-27ºC, infectious virus was not detected on plastic, stainless steel, rubber, alfalfa, wood shavings, straw, corn, swine starter feed, or denim cloth, beyond day zero (Pirtle and Beran, 1996). However, PRRSV can remain infectious for an extended time under specific conditions of temperature, moisture, and pH. PRRSV is stable for months to years at temperatures of -70°C and -20°C.

Approximately 90% of PRRSV infectivity is lost within 1 week at 4°C, but low titers of infectious virus can still be detected for at least 30 days. In solution, PRRSV infectivity persists for 1-6 days at 20-21°C, 3-24 hours at 37°C, and 6-20 minutes at 56°C. The thermal stability of PRRSV in serum and tissues is similar to that described for virus stored in media. PRRSV was isolated from 47%, 14%, and 7% of porcine serum samples stored at 25°C for 24, 48, and 72 hours, respectively. When serum was stored at 4°C or -20°C, PRRSV was isolated from 85% of the samples after 72 hours (Van Alstine et al., 1993). PRRSV is stable at pH 6.5-7.5, but infectivity is rapidly lost at pH below 6 and above 7.5 (Benfield et al., 1992; Bloemraad et al., 1994).

Clinical signs and pathology

Breeding sows infected with PRRSV may develop late-term abortions or farrow an increased number of stillborn pigs, mummified fetuses, and weak live-born piglets (Lager and Halbur, 1996b; Lager et al., 1997; Mengeling et al., 1998; Rossow et al., 1996). Clinical signs in neonatal and nursery pigs include high fever, anorexia, dyspnea, tachypnea, chemosis, conjunctivitis, and reduced growth rates (Halbur et al., 1995, 1996b; Rossow et al., 1994; van der Linden et al., 2003). Growing pigs may exhibit respiratory disease, poor growth performance, and mortality associated with secondary bacterial infections (Collins et al., 1992; Halbur et al., 1995, 1996b; Johnson et al., 2004; Rossow et al., 1994; van der Linden et al., 2003).O

The severity of clinical signs induced by PRRSV has been associated with virus genotype, virus virulence, and host factors i.e. breed and age (Christopher-Hennings et al., 2001; Halbur et al., 1995, 1996a, 1996b; Mengeling et al., 1998; Opriessnig et al., 2002; Thanawongnuwech et al., 1998; van der Linden et al., 2003). North American PRRSV isolates may be linked to more severe clinical disease than European PRRSV (Labarque et al., 2002; van der Linden et al., 2003). Specific differences in pathogenicity among PRRSV isolates have also been reported (Halbur et al., 1996b; Thanawongnuwech et al., 1998). Studies of swine genetics suggest that host genetic components may be involved in pig susceptibility and the immune response to PRRSV (Ait-Ali et al., 2007; Christopher-Hennings et al., 2001; Petry et al., 2005; Vincent et al, 2006).

Pathological lesions associated with PRRSV infection include non-collapsed lungs with multifocal firmness and an apparent tan discoloration in the cranial, middle, accessory lobes and the ventromedial portion of the caudal lobes of the lung (Halbur et al., 1995, 1996b; Rossow et al., 1994). Microscopically, these lesions correspond to lymphohistiocytic interstitial pneumonia characterized by septal thickening with lymphocytes and macrophages, hypertrophy and hyperplasia of type 2 pneumocytes, and accumulation of normal and necrotic macrophages in alveolar spaces (Halbur et al., 1995, 1996b; Rossow et al., 1994). Lymphoid tissues, especially lymph nodes, may be enlarged due to B-cell proliferation in lymphoid follicles, infiltration of macrophages and lymphocytes into the follicles (Halbur et al., 1995, 1996b; Lemke et al., 2004; Rossow et al., 1994). Other lesions reported include rhinitis, encephalitis, and myocarditis (Collins et al., 1992; Halbur et al., 1995, 1996a, 1996b; Rossow et al., 1994).

Immune Response

From earliest first studies, the immune response against PRRSV has been recognized as problematic. In particular, the extended coexistence of virus and antibodies in body fluids suggested atypical or ineffective responses in both humoral and cellular components (Meng, 2000; Ohlinger et al., 1992; van Woensel et al., 1998). This inadequate immune response and the inherent genetic diversity of the virus are that factors primarily contribute to PRRSV persistence (Meng, 2000).

Innate immune is the first barrier against viral infections and a determining factor in the acquired immune response. In the course of the PRRSV infection, the immune response is weak in terms of cytokine production (Royaee et al., 2004; Van Reeth et al., 2002) and the effectiveness of NK cells (Lamontagne et al., 2003; Samson et al., 2000). Many studies have demonstrated that type I interferon (IFNα) and tumor necrosis factor alpha (TNFα) have inhibitory effects on the virus replication. However, levels of type I IFN are minimal or undetectable in serum and bronchoalveolar lavage (BAL) from PRRSV-infected pigs (Albina et al., 1998; Buddaert et al., 1998; Chiou et al., 2000; Labarque et al., 2003; Lopez-Fuertes et al., 2000; Van Reeth et al., 1999). In addition, PRRSV-infected PAMs do not up-regulate TNFα mRNA expression in response to potent TNFα inducers such as phorbol 12-myristate

13-acetate (PMA) plus calcium ionophore (ionomycin) (Lopez-Fuertes et al., 2000). A correspondingly weak response is seen in NK cells. The levels of NK cells in blood or in tissues remain low during the first hours after infection (Lamontagne et al., 2003), with a detectable response five days post-infection (Samson et al., 2000). This weak response is associated with the low levels of IFNα, a potent activator of the NK cells (Murtaugh et al., 2002). Thus, the ineffective immune response against PRRSV could be explained by the weak innate response (Murtaugh et al., 2002; Royaee et al., 2004; Xiao et al., 2004).

Maternal-derived humoral immunity has been demonstrated to be complete for sows and their litters through passive transference of neutralizing antibodies and challenge with the homologous PRRSV isolate (Osorio et al., 2002). However, uncertainties regarding the efficacy of humoral immunity have also been demonstrated (Yoon et al., 1996). The antibody response may only provide partial protection or may be detrimental through antibody-dependent enhancement of PRRSV infection (Yoon et al., 1996).

PRRSV specific antibodies appear rapidly after infection with serum antibodies detectable by 5-7 days post-infection (DPI) and most animals seropositive by DPI 14 (Johnson et al., 2004; Labarque et al., 2000; van der Linden et al., 2003; Yoon et al., 1995). Most antibodies are directed to N protein, the immunodominant epitope (Yoon, et al., 1995), but these antibodies do not confer protection (Labarque et al., 2000, Yoon et al., 1994). PRRSV-specific antibodies are detectable early after infection, with PRRSV-specific immunoglobulin M (IgM) detected in the serum and BAL fluid 9-14 DPI, declining to undetectable levels by 28-42 DPI (Joo et al., 1997; Labarque et al., 2000; Loemba et al., 1996; Park et al., 1995; Vezina et al., 1996). In contrast, IgG levels remain high in serum and BAL fluid for weeks or months (Labarque et al., 2000).

The neutralizing antibody response generally appears approximately 4 weeks after infection and persists at low titers for at least 210 days after infection (Wills et al., 1997b; Yoon et al., 1995; Meier et al., 2003). Neutralizing antibodies are directed against an epitope on GP5 for both American and European genotypes, but also against epitopes on GP4 and M proteins (Diaz et al., 2005; Gonin et al., 1999; Meier et al., 2003; Meulenberg et al., 1997; Weiland et al., 1999; Yang et al., 2000). Neutralizing antibodies can completely deactivate homologous virus, but may not be effective or only partially neutralize heterologous virus

isolates (Bautista et al., 1999; Gonin et al., 1999; Kwang et al., 1999; Lopez and Osorio, 2004; Ostrowski et al., 2002; Plagemann et al., 2002; Weiland et al., 1999; Wissink et al., 2003).

The evidence in support of a protective PRRSV-specific cell-mediated immune (CMI) response is sparse. In inoculated pigs, the infection causes a transient decrease in the number of $CD4^+$ and $CD8^+$ T cells in peripheral blood 3 days after infection (Nielsen and Bøtner, 1997; Rossow et al., 1994; Shimizu et al., 1996). The reduction of $CD4^+$ T cells population lasts at least 2 weeks after infection, whereas the decrease of $CD8^+$ T cells lasts 4 weeks after infection (Lamontagne et al., 2003; Shimizu et al., 1996). Thereafter, the population of CD8+ T cells, mainly $CD4^-CD8^+$ cells increases significantly in peripheral blood and BAL fluid (Lamontagne et al., 2003; Samsom et al., 2000; Shimizu et al., 1996). Primary exposure to PRRSV *in vitro* and *in vivo* induces an increase of IL-10 mRNA expression and IL-10 protein production in PBMC, BAL cells, and BAL fluid (Chung et al., 2003; Labarque et al., 2003; Royaee et al., 2004; Suradhat et al., 2003).

The effect of IFNγ on PRRSV replication has been studied extensively through ELISPOT assay to evaluate the cell mediated immune response. IFNγ-producing PBMC determined by ELISPOT assay had been detected 4 to 8 weeks after PRRSV infection (Foss et al., 2002; Meier et al., 2003; Meier et al., 2004; Royaee et al., 2004). The numbers of IFNγ-producing cells varied from 50-100 cells /10^6 PBMC from 4-10 weeks after infection and gradually increased to approximately 400 cells /10^6 PBMC after 48 weeks of infection (Meier et al., 2003). The IFNγ secretion was mediated primarily by CD4+CD8+ T cells as determined by a marked decrease of IFNγ secretion after complement-mediated depletion of these cells (Meier et al., 2003). The expression of IFNγ in the recall reaction by PBMC of sows infected with PRRSV was significantly correlated with protection against reproductive failure (Lowe et al., 2005). Sows with higher numbers of IFNγ-producing PBMC produced higher numbers of viable piglets at weaning than sows with lower number of IFNγ-producing cells (Lowe et al., 2005).

Recombinant porcine IFNγ had shown to inhibit PRRSV replication in monocytes, PAM, and monkey kidney cell line (MARC-145) (Bautista and Molitor, 1999; Rowland et al., 2001). Piglets infected with PRRSV *in utero* demonstrated a significant increase of IL-6,

IL-10, and IFNγ mRNA expression in their PBMC and BAL cells at the time of farrowing and at 2 weeks of age (Feng et al., 2003; Johnsen et al., 2002). However, no change in IL-12 mRNA expression was observed in BAL cells at 2, 4, and 6 weeks after farrowing (Johnsen et al., 2002).

Viral Persistence

Due to an ineffective immune response, PRRSV produces a chronic, persistent infection in pigs, i.e., infectious virus may be recovered from animal for several months post inoculation. Persistent PRRSV infection has been extensively documented through transmission experiments and by detection of virus in animals. Allende et al. (2000) aptly described PRRSV persistence as a "smoldering" infection in which the virus remains present at low levels in a continuously decreasing percentage of convalescent animals over time. Persistent infection is the key to the virus' success in perpetuating itself within herds and also plays an important role in moving the virus between herds. Persistence is the single most significant epidemiological feature of PRRSV infection.

Recent studies have added to the body of knowledge on persistence. In particular, more recent studies tend to involve larger populations and follow animals for a longer period of time, i.e., Batista et al. (2004) studied persistent infection in 80 4-month old gilts inoculated with PRRSV isolate MN-30-100. Consistent with previous reports, tissue pools (tonsil, superficial inguinal and sternal lymph nodes) from 49 of 50 (98%) animals euthanized 30-100 DPI were PCR positive. Thereafter, 8 of 10 (80%), 3 of 10 (30%), and 2 of 10 (20%) were PCR positive at 110, 120, and 135 DPI, respectively. In an experiment with a similar experimental design, Molina et al. (2005) inoculated 109 2-week-old pigs with ATCC VR-2332, but expanded the monitoring period to 189 DPI. Between 147–189 DPI, approximately 10–30% of tissue samples (tonsil, superficial inguinal and submandibular lymph nodes) were PCR positive.

Transmission

Transmission of PPRSV to susceptible animals may occur through directly contact between pigs or indirectly via an intermediary route. Direct transmission occurs through

intranasal, intramuscular, oral, intrauterine, and vaginal routes of exposure (Benfield et al., 2000; Christianson et al., 1993; Gradil et al., 1996; Magar et al., 1995; Magar and Larochelle, 2004; van der Linden et al., 2003; Yaeger et al., 1993). Indirect transmission includes spreading by fomites (Dee et al., 2002), arthropods (Otake et al., 2002a), and aerosols. Indirect routes of transmission are important because of their role in transmitting PRRSV to neighboring herds (area spread).

The infectivity data indicate that pigs are extremely susceptible to infection via parenteral exposure (breaks in the skin barrier) and much less susceptible by all other routes investigated to date. In the field, potential parenteral exposures include standard husbandry practices, i.e., ear notching, tail docking, teeth clipping, tattooing, and inoculations with medications and biologics. Likewise, because PRRSV is present in saliva for weeks following infection, normal pig behavior commonly results in potential exposures, i.e., bites, cuts, scrapes, and/or abrasions that occur during aggressive interactions among pigs. Bierk et al. (2001) associated transmission with aggressive behavior between carrier sows and susceptible contacts. Other behaviors that result in exchange of blood and saliva, i.e., tail-biting and ear-biting, may also function in transmission.

Infected animals shed virus in saliva (Wills et al., 1997a), nasal secretions (Christianson et al., 1993; Rossow et al., 1994), urine (Wills et al., 1997), semen (Swenson et al., 1994, 1995), and feces (Christianson et al., 1993). Pregnant susceptible females inoculated in late gestation shed virus in mammary secretions (Wagstrom et al., 2001). Shedding of virus in saliva, urine, and feces results in environmental contamination and creates the potential for transmission via fomites, e.g., equipment, instruments, and clothing), substances (e.g., water, food), living carriers (vectors), or aerosols. Otake et al. (2002b) confirmed needle-borne transmission of PRRSV under experimental conditions. Likewise, Otake et al. (2002a) showed that PRRSV was present on workers coveralls, boots, and hands following 60 minutes of contact with acutely infected pigs. Importantly, elementary sanitation procedures, e.g., changing coveralls, changing boots, and washing hands, were sufficient to stop transmission (Dee et al., 2004). Under experimental conditions, Dee et al. (2002, 2003) showed that PRRSV could be moved extensively in the field on fomites in the

field under winter conditions, i.e., below 0°C, but to a much lesser degree during warm weather, i.e., 10-16°C, again illustrating the importance of temperature in virus survival.

Shedding of virus in semen is of particular concern because of the wide-spread use of artificial insemination. The duration of semen shedding varies widely among boars (Christopher-Hennings et al., 1996). Swenson et al. (1994) found infectious virus in the semen of experimentally infected boars for up to 43 days following exposure. By PCR, Christopher-Hennings et al. (1995) detected viral RNA in the semen of experimentally infected boars for up to 92 days post inoculation (DPI) and isolated PRRSV from the bulbourethral gland of a boar euthanized 101 DPI. Semen shedding of MLV vaccine virus occurred for up to 39 days in one study, but prior vaccination eliminated or reduced shedding upon challenge (Christopher-Hennings et al., 1997).

Preliminary reports suggest a possible role for arthropods in PRRSV transmission. PRRSV has been detected in, or on, wild-caught flies and mosquitoes (Otake et al., 2002c; Schurrer et al., 2004). Under experimental conditions, Otake et al. (2003) demonstrated mechanical transmission of PRRSV by mosquitoes and house flies (*Musca domestica*) (Otake et al., 2003). Overall, the current research data suggest that flies and mosquitoes might serve as mechanical vectors of PRRSV. However, the available data have not proven that PRRSV is an arthropod-borne infection in the classical sense.

Airborne transmission, along with arthropod-borne transmission, could explain the apparent long-distance transmission (area spread) of PRRSV in the absence of other sources of virus (pigs, inanimate objects, people), but airborne transmission of PRRSV has been difficult to model. Under experimental conditions, transmission from infected to susceptible pigs over a space of 1.0-2.5 meters has been successful in approximately 50% of the attempts (Lager and Mengeling, 2000; Otake et al., 2002c; Torremorell et al., 1997; Wills et al., 1997b). The one exception to this pattern of poor airborne transmissibility is a report by Kristensen et al. (2004). In three trials, approximately 50 acutely infected pigs transmitted PRRSV over a distance of one meter to approximately 50 susceptible pigs when 1%, 10%, or 70% of air was exchanged. In a field setting, airborne transmission did not occur over distances of 15 meters (Trincado et al., 2004) and 30 meters (Otake et al., 2002a). role of airborne transmission of PRRSV will not be understood until additional information is

available; in particular, the quantity of virus excreted by pigs, the source of the virus, the rate of inactivation of aerosolized virus, and the infectious dose for pigs by aerosol exposure.

PRRSV is transmitted from viremic dams transplacentally to fetuses, resulting in fetal death or birth of infected pigs that are weak or appear normal (Bøtner et al., 1994; Christianson et al., 1992; Terpstra et al., 1991). Some pigs in affected litters may escape infection with PRRSV. PRRSV can replicate in fetuses 14 days of gestational age or older, but infection of fetuses during the first two-thirds of gestation is uncommon because most strains of PRRSV cross the placenta efficiently only in the last trimester of pregnancy (Christianson et al., 1993; Lager and Mengeling 1995; Mengeling et al., 1994; Prieto et al., 1996). The reason for the difference in efficiency of maternal-placental viral transit at different stages of gestation and the mechanism(s) of viral transit are unknown, but is independent of the reproductive virulence of the virus strain. Park et al. (1996) showed that PRRSV strains of low and high virulence for fetuses cross the placenta with equal efficiency when sows were inoculated at 90 days of gestation.

Swine are susceptible to PRRSV by several routes of exposure, including intranasal, intramuscular, oral (Magar et al., 1995; Magar and Larochelle 2004; van der Linden et al., 2003), intrauterine (Christianson et al., 1993), and vaginal (Benfield et al., 2000; Gradil et al., 1996; Yaeger et al., 1993). Pigs are not equally susceptible to PRRSV by all routes of exposure. That is, the probability that a given dose will result in infection differs by route of exposure. Hermann et al. (2005) estimated the infectious dose50 (ID_{50}), i.e., the dose required to infect one-half of the exposed animals, for oral and intranasal routes of exposure to be $1 \times 10^{5.3}$ $TCID_{50}$ and $1 \times 10^{4.0}$ $TCID_{50}$, respectively. Based on data from Benfield et al. (2000), the ID_{50} for exposure via artificial insemination is approximately $1 \times 10^{4.5}$ $TCID_{50}$. Yoon et al. (1999) reported that exposure to 20 or fewer PRRSV particles by intramuscular exposure resulted in infection.

Conclusions and problem to be addressed in the dissertation

PRRSV infection in commercial swine herds is associated with reproductive losses in breeding herds and increased morbidity, mortality, and reduced productivity in growing pigs (Zimmerman et al., 2006). Since the identification of porcine reproductive and respiratory

syndrome virus (PRRSV), it has become endemic in most areas of swine production in the world (Newman et al., 2005; Zimmerman, 2003). Numerous programs have been developed to control and eradicate the disease, but success has been only partial. Management strategies directed at controlling PRRS require a qualitative and quantitative understanding of virus circulation at the level of the population. Therefore, the purpose of this study was to increase our knowledge of PRRSV persistence in populations. The specific objectives of the work described in this dissertation include the following:

1. Derive improved estimates of the virological, immunological, and host correlates of PRRSV persistence and clearance;
2. Evaluate cell-mediated and humoral immune responses to structural and/or non-structural viral proteins on PRRSV-infected pigs over time;
3. Evaluate the diagnostic performance of a commercial PRRSV ELISA in serum and muscle transudate samples and some recombinant protein ELISAs (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) in serum compared.
4. Determine how long PRRSV persisted in tissues and evaluate the potential of PRRSV transmission to naïve pigs via ingestion of PRRSV-contaminated muscle.

These objectives were addressed through the study of PRRSV infection in a population of swine under experimental conditions, as described in the chapters that follow.

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Chapter 2. Viral, immunological, and host correlates of porcine reproductive and respiratory syndrome virus persistence and clearance

A paper to be submitted to Veterinary Research

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ABSTRACT

This experiment was designed as a longitudinal study in which pigs were followed for up to 202 days post inoculation (DPI). On day 0, 109 3-week-old pigs were intramuscularly inoculated with PRRSV isolate VR-2332. Negative control pigs (n = 56) were sham inoculated with minimum essential medium (MEM) by the intramuscular route. Thereafter, at approximately 2-week intervals, serum samples were collected from all animals and a subset of randomly selected animals was euthanized and tissues collected. The presence and amount of virus in tissue and serum was assessed using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), virus isolation, and bioassay. Detection of PRRSV in serum by qRT-PCR showed that most pigs were no longer viremic by 42 DPI, but serum samples from some pigs tested positive up to 154 DPI. Lymphoid tissue was qRT-PCR positive through 202 DPI in one or more pigs at each 2-week sampling point. Infectious virus was recovered from serum and/or lymphoid tissue by virus isolation on MARC-145 cell culture up to 28 DPI. Swine bioassays based on intramuscular inoculation of lymphoid tissue homogenate showed that infectious virus was present in these tissues for up to 175 DPI. More samples were positive for PRRSV by qRT-PCR than other assays, but the discrepancy between qRT-PCR and bioassay results suggested that PCR detected non-infectious virus. Therefore, persistence studies should be based on results from infectivity assays. Overall, the results showed that infectious PRRSV is able to persist in populations for a longer time than previously reported.

1. INTRODUCTION

The persistence of PRRSV infection in swine herds is associated with the ability of PRRSV to evade the immune system and maintain an asymptomatic infection in individual pigs (Allende et al., 2000; Rowland et al., 1999, 2001). PRRSV infection is characterized by the appearance of PRRSV-specific antibodies as early as 7 days post infection (DPI), but neutralizing antibodies and cell-mediated immunity are delayed and, once they appear, are relatively inefficient at clearing the infection. Thus, although the immune system is activated by PRRSV, the infection may be characterized as a chronic persistent infection (Bautista, 1999; Rowland et al., 1999).

Management strategies directed at controlling PRRS require a qualitative and quantitative understanding of virus circulation at the level of the population. Inherent in addressing this problem is the question of how long PRRSV persists in individual pigs. Wills et al. (1997) isolated virus from one of four pigs 154 days post inoculation (DPI). Rowland et al. (2003) isolated virus from tonsil and lymph nodes of pigs infected *in utero* for up to 132 days after farrowing. Horter et al. (2002) detected virus in 10 of 11 animals at day 105 DPI. Allende et al. (2000) detected virus in 2 of 5 pigs at 150 DPI by bioassay. However, these studies also indicated that infection is eventually terminated (Allende et al., 2000; Rowland et al., 2003; Wills et al., 2003). The purpose of this study was to provide an improved estimate of the virological, immunological, and host correlates of PRRSV persistence and clearance.

2. MATERIALS AND METHODS

2.1. Experimental Design

The experiment was designed as a longitudinal study in which samples were collected from pigs and assayed for detection of virus and anti-viral responses over time. Fourteen-day-old (n = 165) PRRSV-negative large white/landrace cross piglets (50% barrows and 50% gilts) were received at the Iowa State University Livestock Infectious Disease Isolation Facility one week prior to the beginning of the experiment. Pigs were derived from 17 litters farrowed on day 162 of a 1000 day production calendar. Only healthy, good-quality piglets were selected for inclusion in the experiment. Pigs were ear-tagged upon arrival such that

individual pigs were randomly assigned to treatments by blindly selecting ear tags. Tag numbers one through 109 (n = 109) were assigned to the PRRSV-inoculated group; numbers 110 through 165 (n = 56) were assigned to the negative control group. Pigs were divided equally by gender between the infected and control groups. Even-numbered tags were assigned to males and odd-numbered tags were assigned to females.

Prior to initiating the experiment, all pigs were tested and determined to be seronegative for PRRSV antibodies using a commercial ELISA (IDEXX Laboratories, Westbrook, ME, USA) and PRRSV-negative status was confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). On day zero, blood samples were collected and pigs were intramuscularly (IM) inoculated with one ml of PRRSV ($1 \times 10^{4.0}$ TCID50) diluted in minimum essential medium (MEM, Sigma Chemical Co., M4655, St. Louis, MO USA). Negative control pigs were sham inoculated with MEM by the intramuscular route.

At approximately 2 week intervals following inoculation, serum samples were collected from all animals and a subset of randomly selected animals was euthanized and tissues collected. On DPI 193, 4 negative control animals and 10 inoculated animals were intramuscularly exposed to the original PRRSV inoculum. Nine of the original inoculates and 4 of the negative controls were left to serve as comparisons. The study was terminated at 203 days post inoculation (DPI).

2.2. Porcine reproductive and respiratory syndrome virus

2.2.1. Virus isolate and propagation

The North American prototype PRRSV, ATCC VR-2332 (American Type Culture Collection, Manassas, VA, USA) was used in the study. The complete genomic sequence for VR-2332 (GenBank® accession number PRU87392) has been published (Nelsen, 1999). The specific virus isolate used in this experiment was derived from a highly homologous plaque-cloned virus (CC-01) described previously (Chang et al., 2002). For this experiment, virus CC-01 was expanded by inoculation into a 21-day-old pig and then propagated on 24 hr-old confluent MARC-145 cells using serum collected at 7 DPI (Kim et al., 1993). MARC-145 cells were prepared in 162 cm^2 flasks (Costar, 3150, Corning Inc., Corning, NY,

USA) containing MEM growth medium: MEM (Sigma Chemical Co., M4655, St. Louis, MO USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, F4922), 50 µg per ml gentamicin (Sigma, G1272), 100 IU per ml penicillin (Sigma, G6784), 100 mg per ml streptomycin (Sigma, G6784), and 0.25 µg per ml amphotericin B (Sigma, A4888). After 24 hr at 37°C in a humidified 5% CO_2 incubator, the MEM growth medium was discarded and the flasks inoculated with 5 ml of serum. After 2 hr at 37°C in a humidified 5% CO_2 incubator, the inoculum was discarded and 50 ml of maintenance medium was added: 50 ml of MEM supplemented with 5% FBS, 50 µg per ml gentamicin, 100 IU per ml penicillin, 100 µg per ml streptomycin, and 0.25 µg per ml amphotericin B. Thereafter, cells were examined daily for cytopathic effect (CPE). When 75% CPE was observed, the medium was freeze-thawed (-80°C / 25°C) and cell lysates were harvested and stored at -80°C.

2.2.2. Virus titration

Virus titrations were done on confluent monolayers of MARC-145 cells in 96-well plates (3596 Corning®, Corning, NY, USA). Cell monolayers were prepared by adding 200 µl of a solution containing 4×10^5 cells per ml suspended in MEM growth medium to each well, after which plates were placed for 24 hr in a 37°C, humidified, 5% CO_2 incubator. Each sample was serially 10-fold diluted in MEM and then 4 wells were inoculated with 100 µl of stock virus at each dilution. Thereafter, plates were incubated at 37°C in a humidified 5% CO_2 incubator for 2 hr, the inoculum was discarded, and 200 µl of maintenance medium was added to each well. Wells were examined for CPE for up to 2 DPI. At the end of 2 days, the cells were fixed with aqueous 80% acetone solution and stained with fluorescein isothiocyanate-conjugated monoclonal antibody (MAb) SDOW17-F (Rural Technologies Inc., Brookings, SD, USA).

2.3. Animal care, handling, and sampling

Experimental design, animal care, and animal handling procedures were approved by both the Biosafety Committee and Animal Care and Use Committee at Iowa State University. In addition, the study was conducted in compliance with the requirements given in the *Guide*

for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 1999).

2.3.1. Serum collection

Blood samples were collected from pigs using a single-use blood collection system (Kendall Company, Manfield, MA, USA), then centrifuged at 1000 × *g* for 10 min. Thereafter, serum was harvested, aliquoted into 2.0 ml cryovials (Fisher Scientific Co.,Hanover Park, IL, USA), and stored at -80°C until tested.

2.3.2. Euthanasia

Five pigs were euthanized at approximately 2-week intervals from DPI 28 through DPI 112; thereafter, approximately 15 pigs were euthanized per sampling point. Pigs to be euthanized were first intramuscularly (IM) administered (1.0 ml per 22.5 kg of body weight) a solution formulated by reconstituting Telazol (250 mg of tiletamine, 250 mg of zolazepam; Fort Dodge Animal Health, Fort Dodge, IA, USA) with 2.5 ml of xylazine (100mg/ml; Lloyd Laboratories, Shenandoah, IA, USA) and 2.5 ml of ketamine (100 mg/ml; Fort Dodge Animal Health). When recumbent, pigs were intravenously administered (1.0 ml per kg of body weight) a solution containing 0.2 mg butorphanol (Fort Dodge Animal Health), 2.0 mg xylazine (Lloyd Laboratories) and 2.0 mg ketamine (Fort Dodge Animal Health). When a surgical plane of anesthesia was reached, pigs were weighed and then exsanguinated.

2.3.3 Lymphoid tissue samples

Strict measures were taken to eliminate cross-contamination of samples with PRRSV at necropsy: 1) negative control pigs were necropsied before PRRSV-inoculated pigs; 2) carcasses were washed with a disinfectant solution prior to beginning necropsy (Pharmacal Research Labs Inc., Naugatuck, CT, USA); 3) necropsy instruments were washed, immersed in methanol, and flamed between each tissue specimen; 4) tissue samples were placed directly into sterile plastic bags (NASCO, Fort Atkinson, WI, USA) 5) specimens were placed on wet ice immediately after collection, frozen (-80°C), and stored frozen until assayed; and 6) latex gloves were changed between each pig. Lymphoid tissues (tonsil,

superficial inguinal and submandibular lymph nodes) were collected and dissected. One section of each tissue was deposited in a sterile 4.0 ml tube (14-959-10 Falcon® Fisher Scientific Co., L.L.C., Hanover Park, IL, USA), the remaining section was placed in sterile plastic bags (Whirl-Pak® Stand-Up Bag; NASCO, Fort Atkinson, WI, USA) and both, stored at -80°C until tested.

2.4. Serum antibody detection

2.4.1 ELISA

A commercial enzyme-linked immunosorbent assay (ELISA) kit (HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Westbrook, ME, USA) was used to detect PRRS virus-specific antibody in serum samples. The assay was performed according to the manufacturer's instructions. A sample was considered positive for PRRS virus antibody if the sample-to-positive (S/P) ratio was ≥ 0.4.

2.4.2. Serum virus neutralization

PRRSV neutralizing activity in serum was measured using a modified fluorescent focus neutralizing assay (FFN). As described by Wu et.al. (2001), two-fold dilutions (1:4 to 1:512) of heat-inactivated pig serum samples were prepared on 96-well plates in MEM (GIBCO, ®, Invitrogen Corporation, Carlsbad, CA, USA) and supplemented with 2% horse serum (Atlanta Biologicals Inc, Lawrenceville, GA, USA). An equal volume of the homologous virus (ATCC VR-2332) at concentration of 2×10^3 $TCID_{50}$ per ml was added to each sample, incubated for one hr at 37°C and then transferred to a 96-well plate containing confluent MARC-145 cells. After 24 hr, the plates were washed, fixed with aqueous 80% acetone and stained with FITC-conjugated monoclonal antibody (MAb) SDOW17 FITC (South Dakota State University, Brookings, SD, USA) diluted 1:100 in PBS. Neutralizing activity was reported as the last dilution that showed ≥ 90% reduction in the number of fluorescent foci.

2.5. PRRSV detection assays

2.5.1. Quantitative, real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

Quantitative, real-time RT-PCR was conducted using a commercial kit (Tetracore Inc., Rockville, MD, USA) as previously described (Wasilk et al, 2004). In brief, RNA extraction from serum was performed using the QIAamp® Viral RNA Mini-Kit (Qiagen Inc., Valencia, CA, USA) following the kit instructions. For RNA extraction from tissues, the sample was weighed and homogenized with an equal volume of phosphate buffer solution (PBS). A guanidinium thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine) was added and RNA was obtained through the Qiagen® Qiashredder™ columns and RNeasy® Mini kit. Quantification of samples was expressed in terms of the number of RNA copies per ml for fluids and copies per gram for tissues. These estimates were based on linear extrapolation of the cycle threshold values against a standard curve generated by serial dilutions of known amounts of *in vitro* transcript RNA product (1×10^{-1} to 1×10^{8}copies per μl).

2.5.2. ORF5 nested reverse-transcriptase polymerase chain reaction (nRT-PCR-ORF5)

PRRSV RNA extraction was performed using the QIAamp® Viral RNA Mini-Kit (Qiagen Inc.) following the manufacturer's protocol. The complete ORF5 sequence, including the ORF 4 and 6 flanking regions, was amplified from the extracted RNA using the Qiagen®OneStep RT-PCR kit (Qiagen Inc.) and two primers based on the sequence of PRRSV isolate VR-2332: P5F (5′- CCT GAG ACC ATG AGG TGG G-3′) and P5R (5′- TTT AGG GCA TAT ATC ATC ACT GG-3′). Reverse transcription and PCR amplification were performed as previously described (Cha et al., 2004). PCR products (764 bp) were purified with a QIAquick® PCR purification kit (Qiagen Inc.) following the kit instructions. The ORF5 nested RT-PCR was performed using 2ul of the final product from ORF5 RT-PCR as template and two primers: PRNE5F (5'-CAA CTG TTT TAG CCT GTC TTT TTG CC-3') and PRNE5R (5'-ACT GGC GTG TAG GTA ATA GA-3'). A positive reaction was confirmed by separating 2 μl of the final product by electrophoresis in a 1% agarose gel (Amresco Inc., Solon, OH, USA) containing 0.1% ethidium bromide in Tris-borate-EDTA buffer and then UV light visualization.

2.5.3. ORF7 nested reverse-transcriptase polymerase chain reaction (nRT-PCR-ORF7)

PRRSV RNA extraction was performed using the RNeasy 96® Universal Tissue Kit (Qiagen Inc) as per the manufacturer's protocol and was tested in a nested RT-PCR assay using methods similar to those published previously (Fetzer et al., 2006). Reverse transcription and the first round of external PCR amplification cycles were performed on the extracted RNA using the Qiagen® OneStep RT-PCR Kit (Qiagen Inc.) and two external primers: PLR (5'-TCGCCCTAATTGAATAGGTG-3') and PLS (5'-ATGGCCAGCCAGTCAATC-3'). The nested RT-PCR was performed using one µL of the first round PCR product and amplified using the Qiagen® Taq PCR Core Kit (Qiagen Inc.) with the two internal primers P-US-7ns (5'-AGTCCAGAGGCAAGGGACCG-3') and P-US-7nas (5'-TCAATCAGTGCCATTCAC CAC-3'). Samples of the second round PCR reactions were visualized using 2% agarose e-gels (Invitrogen Inc.) with image capture under UV light illumination. Results were expressed as positive or negative, with a positive response indicated by the detection of a 337bp PCR product. Hereafter, this is assay is referred to as nPCR-B.

2.5.4. Porcine circovirus 2 (PCV2) polymerase chain reaction (PCR)

PCV2 DNA extraction was performed using the QIAamp Viral DNA Minikit® (Qiagen Inc.) according to manufacture's instructions. Five (5) µl of each extracted serum sample was then assayed for the presence of viral nucleic acid by PCR. The PCR assay was set up as follows: 10µM primer PCV 32 (5'GCAGCACCTCAGCAGCAACA3') and primer 550B (5'GTCTTCCAATCACGCTTCTG 3'), 2mM dNTP's, 1X PCR Buffer, 2.5U Taq DNA Polymerase and 5 µl/reaction of extracted serum DNA. The samples were run in a thermocycler as follows: 94°C 5 minutes, 35X (94°C 30 sec, 56°C 30 sec, 72°C 45 sec), 72°C 10 minutes. After the PCR assay, the samples were electrophoresed on an agarose gel with ethidium bromide and the results visualized using an UV light box. A positive PCR reaction produced a PCR product of 518bp for either PCV type1 or PCV type 2, if the serum sample contained either of these target viruses.

2.5.5. PRRSV isolation

Virus isolation was performed following the virus titration protocol described in Section 2.2.2., except that 50 µl of serum or clarified tissue homogenate was inoculated onto 4 wells of a 96-well plate, after which plates were incubated at 37°C in a humidified 5% CO_2 incubator for one hour. Thereafter, the inoculum was discarded and 200 µl of maintenance medium was added to each well. Plates were incubated for an additional 5 days at 37°C in a humidified 5% CO_2 incubator. After 4 days, inoculated cells were frozen, thawed, and a second pass was made by adding 200µl of each well to another plate of confluent monolayer of MARC-145 cells. The presence of virus was determined by microscopic observation for typical cytopathic effects. Subsequently, cells were washed with PBS, fixed with 80% acetone solution, and stained with fluorescein isothiocyanate-conjugated a PRRSV-specific monoclonal antibody (FITC-SDOW-17, Rural Technologies Inc.).

2.5.6. Swine bioassay

Lymphoid tissues collected at necropsy from 96 of the PRRSV-inoculated pigs were tested for infectious PRRSV by swine bioassay. Each lymphoid tissue specimen (tonsil, submandibular lymph node, superficial inguinal lymph node) was processed separately. To prepare the homogenate, 2 grams of minced tissue were mixed with 8 ml of MEM (Sigma Chemical) supplemented with 5% FBS (Sigma Chemical) without antibiotics, macerated for a minimum of 2 min (Stomacher®80 Biomaster, Fisher Scientific) and centrifuged (4,500 × *g*) for 20 min. The supernatant was harvested and administered to bioassay pigs the same day the homogenate was prepared.

Three-week-old PRRSV-naive bioassay pigs were individually housed under biosafety level 2 conditions. Each recipient pig was inoculated IM with 7 ml of supernatant prepared from tonsil, 7 ml prepared from submandibular lymph node, and 7 ml prepared from superficial inguinal lymph node. Each tissue homogenate was inoculated at a different site on the bioassay pig. Following inoculation, serum samples were collected on 0, 5 and 10 DPI and tested by qRT-PCR for evidence of PRRSV infection. A positive qRT-PCR result indicated that the tissue homogenate had contained infectious PRRSV.

2.6. PRRSV ORF 5 sequencing

Viruses selected for ORF5 sequencing included 20 plaque-cloned viruses recovered from the inoculum (CC-01-04) used to inoculate pigs on day zero and 14 qRT-PCR-positive serum samples collected 10 days after inoculation of bioassay pigs. Plaque-cloned viruses from CC-01-04 were sequenced to provide a measure of the PRRSV genetic variability in the original inoculum. Plaque-cloning was done following a procedure described previously (Cha et al., 2004; Chang et al., 2002). In brief, the sample was inoculated into one well of a six-well plate containing confluent MARC-145 cells. After 2 hrs, the inoculum was removed and replaced with 5 ml of overlay media composed of 1% agarose (Sigma) and DMEM supplemented with 10% fetal bovine serum, gentamicin, and amphotericine B. The cells were incubated for 3 days at 37^{o}C in humidified 5% CO_2 incubator, after which well-demarcated plaques were selected, suspended in 0.5 ml of culture medium, and propagated in MARC-145 cells. A total of 20 plaque-cloned viruses were recovered. Day 10 serum samples from bioassay pigs were sequenced to provide a measure of virus evolution over the course of the experiment.

For sequencing, RNA was extracted using the QIAamp® Viral RNA Mini-Kit (Qiagen Inc.) following the manufacturer's protocol. The complete ORF5 sequence, including the ORF 4 and 6 flanking regions, was amplified from the extracted RNA using the Qiagen®OneStep RT-PCR kit (Qiagen Inc.) and two primers based on PRRSV isolate VR-2332 sequences: P5F (5′- CCT GAG ACC ATG AGG TGG G-3′) and P5R (5′- TTT AGG GCA TAT ATC ATC ACT GG-3′). Reverse transcription and PCR amplification were performed as previously described (Cha et al., 2004). PCR products (764 bp) were purified with a QIAquick® PCR purification kit (Qiagen Inc.) following the kit instructions. The final purified RT-PCR products were sequenced with P5F (5′- CCT GAG ACC ATG AGG TGG G-3′) and P5R (5′- TTT AGG GCA TAT ATC ATC ACT GG-3′) primers at the Iowa State University Nucleic Acid Facility. To compare sequence homology, multiple sequence alignment was done using sequence analysis software (Lasergene®, DNASTAR Inc., Madison, WI, USA). Unrooted phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA software 3.1 (The Biodesign Institute, Tempe, AZ, USA). Bootstrap values were calculated on 1000 replicates of the alignment.

2.7. Cytokine protein analysis

Interleukin (IL) 1b, IL8, IL10, and interferon gamma (IFNγ) was quantified in sequential serum samples collected at 14 day intervals from 20 negative control and 54 PRRSV-inoculated animals. Interleukin (IL) 1b, IL8, IL10 were evaluated in samples collected through DPI 42 and IFNγ in samples collected through DPI 84. Serum samples were assayed using commercial ELISA kits (IL1b and IL8: R&D Systems, Minneapolis, MN, USA; IL10 and IFNγ: Biosource™ Invitrogen, Camarillo, CA, USA) following the protocols provided by the manufacturers, except that the IFNγ (Biosource™ Invitrogen) standard curve was taken to a maximum of half the suggested level to save standard and to allow for detection of lower amounts of cytokine in the dynamic range of the assay (maximum optical density (OD) = 2.0 for the lower concentration). The substrate, SureBlue™ (KPL, Inc., Gaithersburg, MA, USA), was added and plates were read at 650nm until the highest standard reached an OD of ~2.0. No acid was added. For all ELISA data, the OD of medium controls was averaged and subtracted from the OD of each sample and then the lowest readable standard concentration was determined from the standard curve. If the OD was below the lowest part of the curve, values were assigned by dividing the lowest readable standard concentration by 2 and then multiplying that number by the dilution factor (i.e. 2, 4, or 10).

2.8. Immune gene expression

Immune gene expression profile in response to PRRSV was monitored by real-time RT-PCR on RNA and cDNA prepared from tracheobronchial lymph nodes (TBLN). cDNA was assayed for expression of a panel of 10 genes involved in the development and regulation of immunity: IFNα, IFNγ, IL1, IL5, IL6, IL8, IL10, IL12b, IL13, IL15, and tumor necrosis factor (TNF). RNA was extracted from TBLN with Trizol® (Invitrogen, Carlsbad, CA, USA) and its integrity and quantity was assessed using the Agilent Bioanalyzer 2100 and RNA 6000 Labchip® kit (Agilent Technologies, Palo Alto, CA USA). All RNAs were DNAse-treated prior to cDNA synthesis using Superscript™ Reverse Transcriptase (Invitrogen Inc.) and oligo-dT primers (Invitrogen Inc.), as previously described (Dawson et

al., 2005). Real-time PCR was performed on 100 ng cDNA (RNA equivalent) per 25 µl reaction per well using the Stratagene® Brilliant kit (La Jolla, CA USA) and an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). All probes and primers for real-time PCR were designed using the Primer Express® (Applied Biosystems) software package and nucleotide sequences obtained from GenBank® or the TIGR porcine EST database (Institute for Genomic Research, Rockville, MD, USA) to generate amplicons spanning adjacent exons when possible (Dawson et al., 2005). Relative quantification of target gene expression was evaluated using cycle threshold (C_t) values. Gene expression data were normalized to the amount of RNA per cDNA amplified (Bustin, 2002).

2.9. Swine Leukocyte Antigen (SLA) Class I Characterization.

Total DNA for swine leukocyte antigen (SLA) characterization was recovered from lymphoid tissues (superficial inguinal and submandibular lymph nodes) using the Qiagen® DNeasy Blood and Tissue kit spin-column protocol (Qiagen Inc.) following the instructions provided by the manufacturer. Swine leukocyte antigen (SLA) class I types were determined in 101 of the 109 PRRSV-inoculated pigs using a "one-step" PCR approach (Ho et al., unpublished data). Briefly, a set of sequence-specific PCR primers were designed to differentiate class I alleles by groups with similar sequence motifs (i.e., low-resolution SLA typing). Typing was performed on a standard 96-well PCR plate, with two animals typed on each plate. PCR reactions, thermal cycling parameters, and gel electrophoresis were performed as previously described (Ho et al., 2006; Martens et al., 2003). Alleles of the swine leukocyte antigen (SLA) class Ia loci (SLA-1, SLA-2 and SLA-3) for which DNA sequencing was available were assigned to allele groups by the SLA Nomenclature Committee on the basis of sequence similarity (Smith et al., 2005). Because of the unavailability of pedigrees, the SLA haplotypes in this population were deduced from the class I haplotypes previously identified in nearly 700 pedigreed pigs obtained from multiple commercial sources using the same typing method (Ho et al., unpublished data). Ambiguities were resolved on the basis of the presence of SLA homozygous animals within

the population, comparisons between different genotype combinations, and associated class II haplotypes determined by a similar typing method.

2.10. Statistical analyses

Data were analyzed using MedCalc® 9.2.1.0 (MedCalc Software, Mariakerke Belgium), and SigmaPlot® 10 (Systat Software, Inc., Richmond, CA, USA). Logarithmic transformation of qRT-PCR and FFN values was performed to normalize values prior to the analyses.

Initially, descriptive and comparative analyses were conducted to describe the immune responses against PRRSV (DPI 0 to 202). Pigs were categorized as persistently infected by the detection of infectious PRRSV in any sample collected at the time of euthanasia. The magnitude and duration of viremia (qRT-PCR) and humoral immune responses (ELISA and FFN) were evaluated using survival curves (Kaplan-Meier method). Differences between groups of persistently infected vs not persistently infected pigs, and the effect of possible covariates on the survival curves, were analyzed by Cox proportional-hazard regression. Repeated measures data for each pig, i.e., ELISA, FFN, qRT-PCR and cytokine proteins in serum, were summarized as the cumulative area under the curve (AUC) using the trapezoidal rule. Thereafter, AUCs were treated as continuous variables and analyzed using one way ANOVA for overall differences between groups, as well as in the multivariate analyses. Multiple logistic regression was used to evaluate the relationship between virus load (virus magnitude and virus duration) and immune responses to PRRSV persistence. Multiple regression analysis was used to examine the immune response and level of viremia.

3. RESULTS

All pigs were determined to be free of PRRSV infection upon arrival into the animal holding facilities at DPI (-)7 and again on DPI 0 by qRT-PCR and ELISA analysis of serum samples. All PRRSV-inoculated pigs were confirmed to be infected on the basis of qRT-PCR-positive results on serum samples collected on DPI 7 and 14. All negative control animals remained free of PRRSV infection throughout the course of the experiment. In

addition, pigs were confirmed to be free of PCV2 infection on the basis of PCR analysis of serum samples collected from 10 infected pigs and 10 control pigs at DPI 0, 7, 14, and 28.

3.1. Body weight

Body weights were recorded at the time individual pigs were euthanized (Table 1, Figure 1). The mean body weight of PRRSV-infected pigs was less than that of the control pigs at every sampling point. A comparison of means by DPI showed that PRRSV-infected pigs weighed significantly less than controls at DPI 112, 119, 133, and 189 (t-test; $p < 0.05$). Consistent with this observation, the average daily gain was significantly different between the two groups, e.g., 0.68 ± 0.18 kg in the infected group vs. 0.86 ± 0.11 kg in the control group (t-test; $p < 0.0001$).

To identify factors that could predict the reduction in body weight, pigs were classified as "light", i.e., ≥ 1 standard deviation lower than the mean at the time of euthanasia, or "normal". Logistic regression analysis showed that longer duration and greater magnitude of viremia (qRT-PCR) was significantly associated with light pigs ($p = 0.03$), but the strength of the association was weak, i.e., odds ratio 1.02 (95% CI= 1.00 to 1.03).

Other factors with possible effect on the determination of the infectious PRRSV persistence, cytokines expression (IFNγ, IL12B, IL15, IRF1, CTIIA, TNF, NOS2A, CXXL10, IL13, IL5, IL10, CASP1, CASP3, CASP8, TAP2, TGM3,) were included in a model of multiple logistic regression; however, none of the variable analyzed had a detectable effect on the body weight. The same cytokines were included in a model of multiple regressions. Analysis results, indicated that genetic expression of interferon (INF) γ in lymph nodes had a significant effect (coefficient = 31.41, t = 2.63) on the variation on body weight. Similarly, levels of tumor necrosis factor (TNF), were significantly associated (coefficient = -56.07, t = - 3.41) with the body weight. (Table 6)

Table 1. Summary by DPI of body weighs (kg)

		Infected group		Control group	
DPI[a]	Pigs (n)	Mean	SD	Mean	SD
42	3	25.25	1.89	27.44	1.60
56	3	30.09	3.96	36.06	8.66
70	3	38.72	14.43	48.08	8.40
84	3	43.54	13.81	60.10	2.89
98	3	60.03	16.14	77.34	3.53
112	3	70.76	19.28	99.11	12.51
119	11	85.73	11.23	102.06	4.56
133	10	98.43	14.91	117.03	5.84
147	10	116.35	10.48	126.92	12.22
161	10	139.21	12.41	114.33	16.30
175	10	151.82	21.76	157.40	12.31
189	10	148.96	20.50	173.09	10.78
202	9	153.87	11.97	163.57	20.89

[a] Days post inoculation
[b] Body weight recorded at euthanasia date

Figure 1. Body weight collected at euthanasia, Scatter plot of individual pig weights and linear regression analysis.

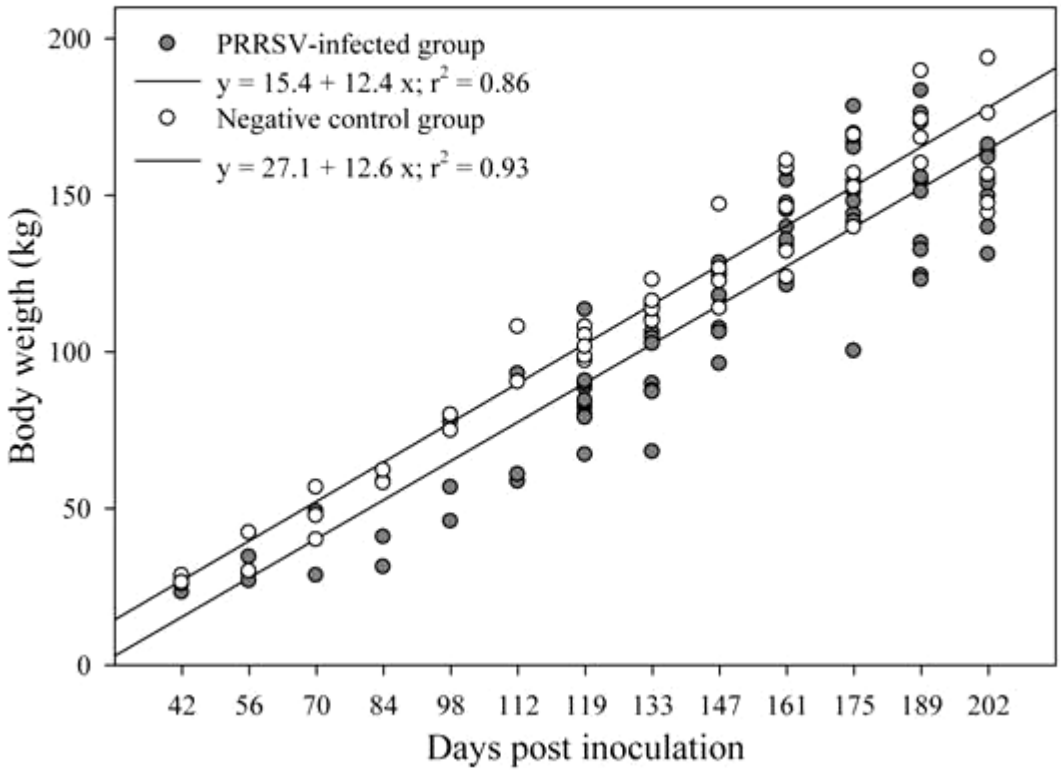
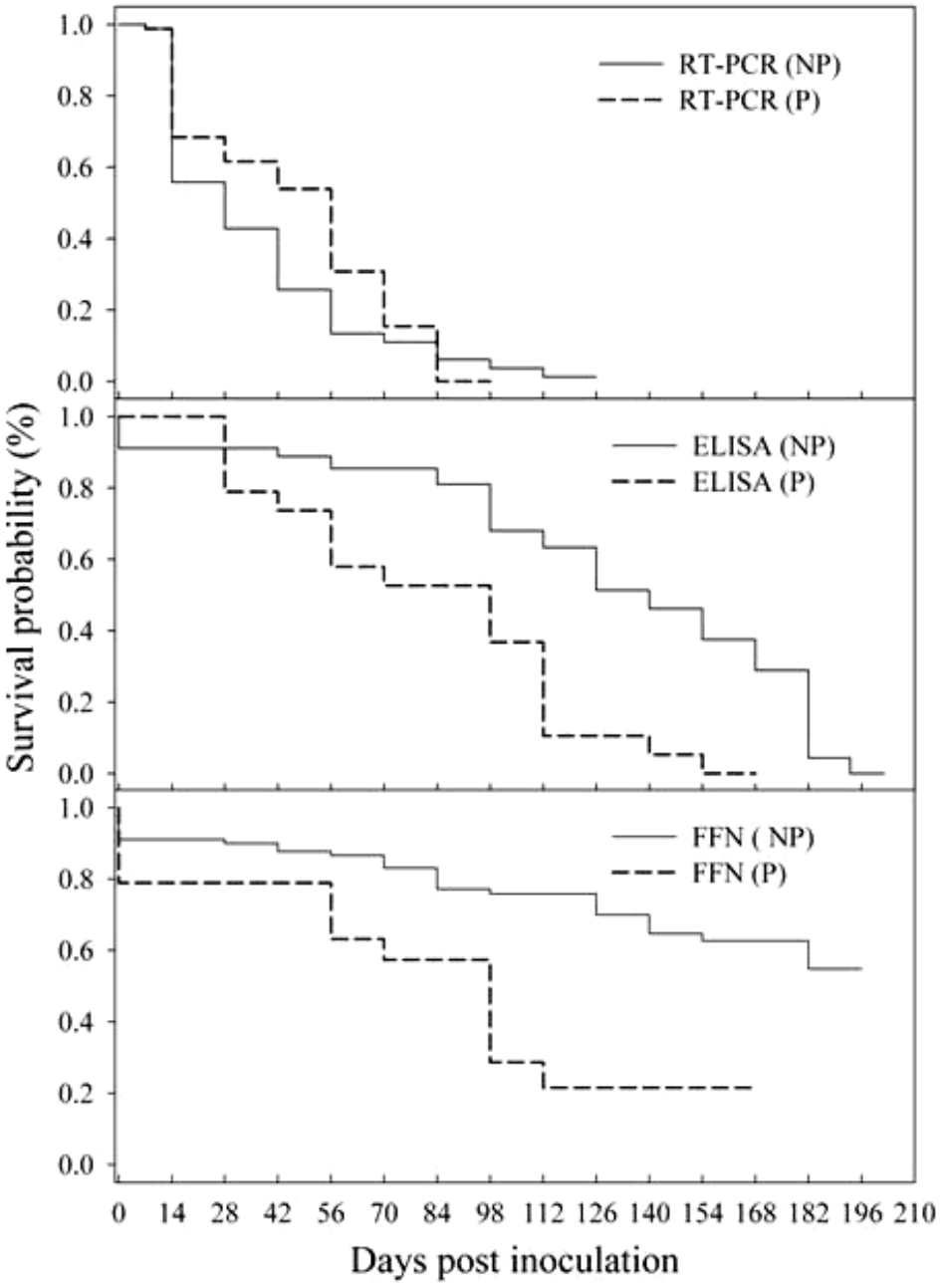


Figure 2 . Survival analysis curves to compare RT-PCR, ELISA and FFN results between group of persistent and no persistent pigs with infectious PRRSV (estimated by VI or bioassay).



NP = No PRRSV persistent
P = PRRV Persistent

3.2. Serum antibody detection

PRRSV-specific antibody response was measured using a commercial ELISA (IDEXX Laboratories) (Table 2, Fig 2). Using the manufacturer's recommended cut-off (S/P ≥ 0.4), all pigs were seronegative for PRRSV antibody at DPI 0 (mean S/P = 0.03; range: 0.00 - 0.12) and all negative-control pigs tested negative throughout the study (mean S/P = 0.02; range: 0.00 - 0.09). The first positive results appeared at 7 DPI (4 of 109 pigs). The peak ELISA response occurred on DPI 42 when 98% (99 of 101) of pigs tested positive with a mean S/P value of 2.02 (95% CI 1.87 - 2.16).

Figure 3. PRRS ELISA serum antibody response. (1) S/P values means and standard error by DPI. (2) ELISA-positive pigs (%) by DPI

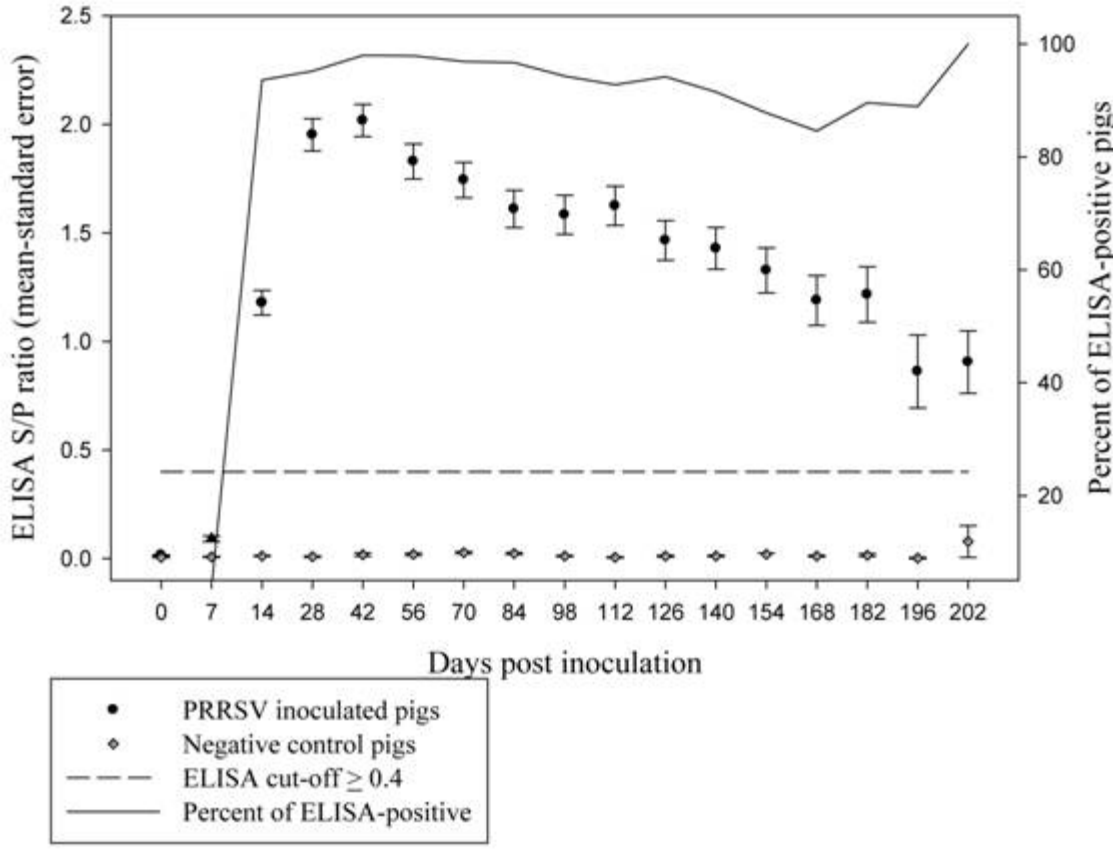


Table 2. Summary of PRRS virus detection by different assays.

		PRRSV qRT-PCR				PRRSV Virus Isolation					Swine Bioassay
DPI [a]	Serum	LT [b]	Tonsil	Sub mand	Sup inguinal	Serum	LT [b]	Tonsil	Sub mand	Sup inguinal	LT [b]
28	3/3	3/3	3/3	3/3	3/3	3/3	2/2	2/2	1/2	2/2	1/3
42	2/3	3/3	3/3	3/3	2/3	0/3	2/3	1/3	2/3	2/3	1/3
56	0/3	3/3	3/3	3/3	2/3	1/3	0/3	0/3	0/3	0/3	2/3
70	2/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	3/3
84	0/3	3/3	2/2	2/3	2/3	0/3	0/3	0/3	0/3	0/3	1/3
98	0/3	3/3	3/3	2/2	0/3	0/3	0/3	0/3	0/3	0/3	0/3
112	0/3	1/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3
119	0/11	10/10	5/10	8/10	6/10	0/11	0/10	0/10	0/10	0/10	3/11
133	0/9	7/9	6/10	5/10	4/10	0/9	0/9	0/9	0/9	0/9	5/9
147	0/10	8/10	3/10	4/10	7/10	0/10	0/10	0/10	0/10	0/10	0/10
161	0/10	7/10	3/10	2/10	4/10	0/10	0/10	0/10	0/10	0/10	1/10
175	0/10	5/10	2/10	5/10	2/10	0/10	0/10	0/10	0/10	0/10	1/10
189	0/10	4/10	1/10	2/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
202	0/9	2/9	1/9	0/9	1/9	0/9	0/9	0/9	0/9	0/9	0/9

[a] Days post inoculation
[b] Lymphoid tissue

Serum FFN results are reported in Table 5. Based on a cut-off of ≥1:4, all pigs, with the exception of two pigs with a titer of 1:4, were seronegative for PRRSV at day 0 and all negative-control pigs remained negative throughout the study.

Neutralizing antibodies were first detected at DPI 7 (1/109 pigs). The peak response appeared at DPI 56 (90/97 pigs) with a median titer of 1:8 (range ≤ 1:2 to 1:32). At the last sampling (DPI 202), 7 of 9 pigs tested positive. However, the neutralizing antibody response was heterogeneous among PRRSV- inoculated pigs. Some pigs developed stronger neutralizing response, whereas others developed detectable response after 70 DPI.

Table 3. Summary of RT-PCR results on serum of pigs from the inoculated group.

DPI	Pigs (n)	Mean	95% CI	Percent of positive
0	109	Negative	Negative	0%
7	109	$1 \times 10^{6.9}$	$1 \times 10^{6.7 \text{ to } 7.1}$	100%
14	105	$1 \times 10^{6.8}$	$1 \times 10^{6.6 \text{ to } 7.1}$	100%
28	105	$1 \times 10^{4.7}$	$1 \times 10^{4.4 \text{ to } 4.9}$	98%
42	101	$1 \times 10^{0.9}$	$1 \times 10^{0.7 \text{ to } 1.3}$	37%
56	97	$1 \times 10^{0.6}$	$1 \times 10^{0.3 \text{ to } 0.8}$	23%
70	94	$1 \times 10^{0.4}$	$1 \times 10^{0.2 \text{ to } 0.6}$	18%
84	91	$1 \times 10^{0.2}$	$1 \times 10^{0.0 \text{ to } 0.3}$	4%
98	86	$1 \times 10^{0.2}$	$1 \times 10^{0.0 \text{ to} 0.3}$	7%
112	82	$1 \times 10^{0.03}$	$1 \times 10^{0.0 \text{ to } 0.0}$	2.4%
126	69	$1 \times 10^{0.03}$	$1 \times 10^{0.0 \text{ to } 0.1}$	2.9%
140	59	Negative	Negative	0%
154	47	$1 \times 10^{0.04}$	$1 \times 10^{0.0 \text{ to } 0.1}$	2.0%
168	33	Negative	Negative	0%
182	23	Negative	Negative	0%
196	10	Negative	Negative	0%
202	9	Negative	Negative	0%

Table 4. Summary of ELISA[1] on serum

	Inoculated group				Negative control group			
DPI	Pigs (n)	Mean	95% CI	Percent of positive	Pigs (n)	Mean	95% CI	Percent of positive
0	109	0.016	0.01, 0.02	0.0%	56	0.006	0.00, 0.01	0.0%
7	109	0.091	0.06, 0.12	3.7%	55	0.009	0.01, 0.01	0.0%
14	105	1.178	1.07, 1.29	93.6%	54	0.013	0.01, 0.02	0.0%
28	105	1.952	1.80, 2.10	95.2%	54	0.009	0.00, 0.02	0.0%
42	101	2.016	1.87, 2.16	98.0%	53	0.018	0.00. 0.03	0.0%
56	97	1.829	1.67, 1.99	97.9%	50	0.019	0.01, 0.03	0.0%
70	94	1.743	1.58, 1.90	96.9%	48	0.028	0.02, 0.04	0.0%
84	91	1.576	1.44, 1.78	96.7%	45	0.023	0.02, 0.03	0.0%
98	87	1.509	1.40, 1.76	94.3%	43	0.012	0.01, 0.02	0.0%
112	83	1.597	1.44, 1.80	92.8%	41	0.006	0.00, 0.01	0.0%
126	69	1.437	1.28, 1.65	94.2%	34	0.013	0.01, 0.02	0.0%
140	59	1.440	1.20, 1.60	91.5%	29	0.015	0.01, 0.02	0.0%
154	49	1.291	1.12, 1.54	87.8%	23	0.019	0.01, 0.03	0.0%
168	39	1.139	0.96, 1.42	84.6%	18	0.012	0.01, 0.02	0.0%
182	29	1.148	0.88, 1.41	89.6%	12	0.017	0.00, 0.03	0.0%
196	9	0.861	0.47, 1.25	88.9%	4	0.005	0.00, 0.01	0.0%
202	9	0.905	0.57, 1.24	100.0%	4	0.079	0.15, 0.31	0.0%

[1] HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Westbrook, ME, USA

Table 5. Summary of quantitative results of FFN

	Inoculated group					
DPI	Pigs (n)	Mean	95% CI	Median	95% CI	Percent of positive
0	109	0.04	-0.01, 0.09	0.00	0.00, 0.01	1.8%
7	109	0.00	0.00, 0.00	0.00	0.00, 0.00	0.0%
14	105	0.01	-0.01, 0.02	0.00	0.00, 0.00	0.9%
28	105	0.08	0.00, 0.15	0.00	0.00, 0.00	3.8%
42	101	1.71	1.43, 1.99	2.00	2.00. 2.00	64.4%
56	97	2.70	2.47, 2.93	3.00	2.00, 3.00	92.8%
70	94	2.03	1.73, 2.33	2.00	2.00, 2.00	73.4%
84	91	1.66	1.37, 1.94	2.00	2.00, 2.00	65.9%
98	87	1.47	1.20, 1.74	2.00	0.91, 2.00	62.1%
112	83	2.51	1.60, 3.41	0.00	0.00, 4.00	46.4%
126	69	1.17	0.91, 1.43	0.00	0.00, 2.00	55.7%
140	59	1.92	1.62, 2.23	2.00	2.00. 2.00	79.3%
154	49	1.90	1.55, 2.25	2.00	2.00. 2.66	75.5%
168	39	1.67	1.24, 2.09	2.00	0.21. 2.00	66.7%
182	29	1.86	1.39, 2.33	2.00	2.00. 3.00	75.0%
196	9	1.89	0.99, 2.79	2.00	0.00, 3.00	77.8%
202	9	2.56	1.33, 2.79	2.00	0.00, 3.00	77.8%

3.3. PRRSV detection

3.3.1. qRT-PCR assays

Summary of qRT-PCR results on serum samples is presented in Tables 2 and 3. Overall, all serum samples from PRRSV-inoculated pigs were qRT-PCR-positive on DPI 7, 14, and 28. Thereafter, the percent of PRRSV-positive serum samples and the level of viremia declined quickly over time. Last qRT-PCR positive serum sample was collected on DPI 154.

A summary of qRT-PCR results on lymphoid tissue (submandibular lymph nodes, superficial inguinal lymph node and tonsil) is presented in Table 2 and 3. Statistical analysis was performed by paired comparison among the 3 types of lymphoid tissues tested with

qRT-PCR, was performed using McNemar test. Submandibular lymph nodes samples were not significant different ($p = 0.58$) from either superficial inguinal lymph nodes or tonsil ($p = 0.54$); superficial inguinal lymph node were not significant different ($p = 0.85$) from tonsil samples. Thereafter, a cumulative data from the 3 types of lymphoid tissue was used to compare the qRT-PCR with two nested PCR (nPCR) based on ORF5 and ORF7, respectively.

In addition, paired comparisons of qualitative results obtained from the qRT-PCR, ORF5-nPCR, and ORF7-nPCR used to test the presence of PRRSV RNA in lymphoid tissues were performed using McNemar test. The analyses indicated that results of the ORF5-nPCR and ORF7-nPCR assays were not significantly different in the percent of paired samples detected ($p = 0.58$); however, qRT-PCR were significant different in the percent of paired samples detected when comparing ORF5-nPCR and ORF7-nPCR ($p < 0.0001$).

To evaluate the ability of the different PCRs (ORF5-nPCR, ORF5-nPCR and qRT-PCR) to detect infectious virus, the result from each test was compared with the outcome from the bioassay using McNemar test. Results from qRT-PCR were significant different in the percent of paired samples detected from bioassay ($p < 0.0001$). In contrast, ORF7-nPCR ($p = 0.27$) and ORF5-nPCR ($p = 1.00$), were not significantly different from bioassay. The best performance was observed when using the ORF7-nPCR, i.e., results produced by this test differed 2.2% of the time compared with bioassay.

The effect of the magnitude and duration of viremia on the strength of the humoral response was evaluated by simple regression of qRT-PCR area under the curve (AUC) and ELISA and FFN responses. These analyses indicated that the relationship between viremia and the strength of the humoral response is too weak to explain either the strength of the ELISA response ($r^2 = 0.0001$, $p = 0.91$) or the FFN response ($r^2 = 0.004$, $p = 0.53$).

3.3.2. Virus isolation

Virus isolation was attempted on the samples that were assayed by qRT-PCR (serum, lymphoid tissue). Summary of the number of positive samples from which PRRSV was recovered is presented in Table 2. In brief, PRRSV was isolated from serum at DPI 28, and DPI 56 and from lymphoid tissue at DPI 28 and DPI 42. No virus was isolated from any

tissue after DPI 56. With the exception of one serum sample collected at DPI 56, the samples from which virus was isolated were also positive by qRT-PCR, but many qRT-PCR positive samples were VI negative (Table 2)

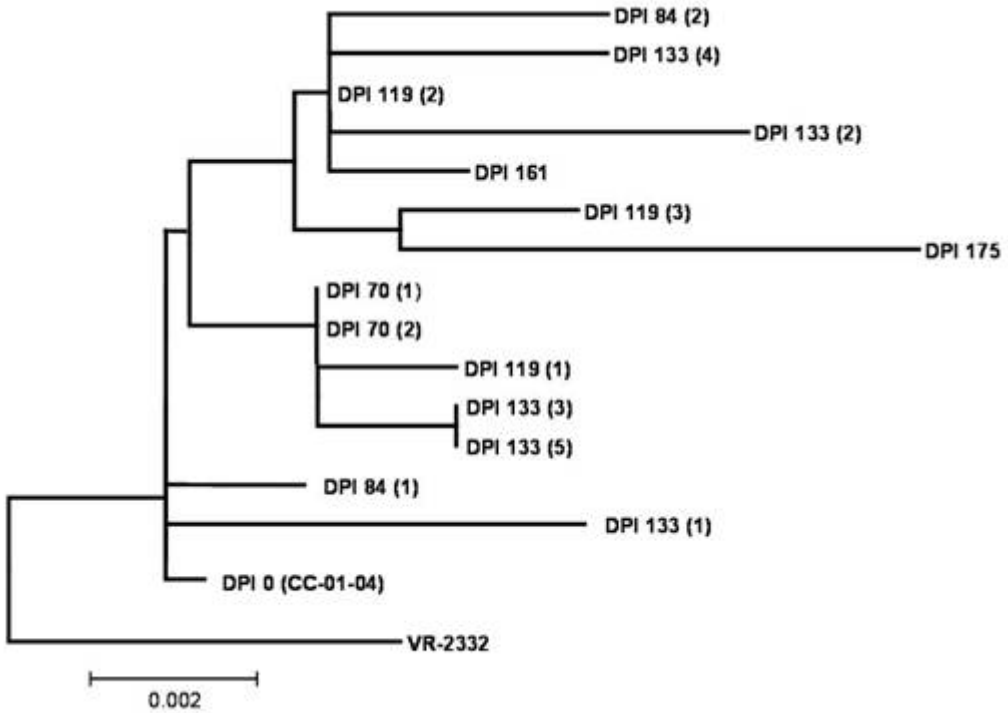
3.3.3. Bioassay

Bioassays were conducted by inoculating pigs with lymphoid tissue homogenate prepared from tonsil, submandibular lymph node, and superficial inguinal lymph node recovered from 96 of the 109 principle pigs. The presence of infectious PRRSV in tissue homogenate was indicated by the detection of PRRSV RNA by qRT-PCR in serum of bioassay pigs following exposure. A summary of the distribution of positive pigs over time is presented in Table 2. In brief, infectious PRRSV was detected in 17 of 96 (16.5%) of the pigs, with 1 of 10 bioassay pigs was positive at both DPI 161 and DPI 175.

3.4. PRRSV sequencing

To measure the genetic changes in PRRSV over the course of the study, isolates from 70 DPI to 175 DPI were sequenced (ORF5) and compared to the original virus inoculum. To avoid biases associated with selection based on the virus' ability to propagate in cell culture, sequencing was done directly from serum samples collected 10 days after inoculation of bioassay pigs with lymphoid tissue homogenate. To serve as a basis of comparison and as a measure of the viral genetic variability in the original inoculum, 20 plaque-cloned viruses from inoculum CC-01-04 were also sequenced. As shown in Figure 4, the nucleotide (amino acid) divergence of ORF5 among the PRRSV recovered after different days post inoculation, ranged from 0.001 to 0.01, showing a high degree of homology with the original inoculum (CC-01-04).

Figure 4. Phylogenetic relationship of PRRSV isolates at different sampling points with the North American PRRSV isolate, with the original inoculum (CC-01-04) and among them based on ORF5 sequence.



Unrooted phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA software 3.1 (The Biodesign Institute, Tempe, AZ, USA).

3.5. Survival Analysis

Survival curves of the duration of viremia, indicated by the detection of PRRSV RNA using qRT-PCR is presented in Figure 2. The median survival time was 42 DPI (95% CI: 28, 42). Survival curves between persistent and not-persistent pigs indicated that median survival in persistent pigs was 70 DPI and median survival in group of not-persistent pigs was 42 DPI. The log rank test indicated that the trend between groups was different (p = 0.12) with a hazard ratio of 1.44. Cox proportional/hazard regression indicated a not significant difference between groups of NP and P (p = 0.69), with a risk ratio ($Exp^{(b)}$= 0.69) and parameter estimates (CI = 0.8-1.25). Analysis of possible cofactors on the survival response by Cox proportional- hazard regression, indicated that neither the ELISA nor the

neutralizing antibody (FFN) response, as estimated by AUC and peak of response, contributed significantly to predict the survival time.

Survival curves of the duration of the humoral response, as measured by ELISA, are presented in Figure 2. The median survival time was 140 DPI (95% CI: 126, 140). Survival curves between persistent and not-persistent pigs indicated that median survival in group of persistent pigs was 112 DPI and median survival in group of not-persistent pigs was 140 DPI. Log rank test indicate that the trend between groups was different ($p < 0.0001$), with a hazard ratio of 0.36. Cox proportional/hazard regression indicated a significant difference between groups of persistent and not-persistent pigs ($p < 0.0001$) with a risk ratio ($Exp^{(b)}$= 3.07) and parameter estimates (CI = 1.78, 5.28).

Analysis of the effect of possible cofactors on the survival response by Cox proportional / hazard regression indicated that virus load, estimated by AUC of repeated measures of qRT-PCR, had no effect over the duration of ELISA response ($p = 0.84$). Neutralizing antibodies (FFN), estimated by AUC, contributed significantly to predict the duration of ELISA response ($p < 0.0001$) and was a covariate in the difference between groups of persistent and not-persistent pigs ($p< 0.0001$).

Survival curves of the duration of neutralizing antibody response estimated using FFN is presented in Figure 2. The median survival time was 196 DPI. Survival curves of persistent and not-persistent pigs indicated that median survival in persistent pigs was 98 DPI; however, the median survival in group of not-persistent pigs was estimated more than the period of this experiment (202 DPI). Weibull estimates indicated that FFN log2 transformed data did not fit an exponential distribution.

To evaluate the possible effect of other factors on the duration of viremia, cytokine responses (IFNG, IL12B, IL15, IRF1, CTIIA, TNF, NOS2A, CXXL10, IL13, IL5, IL10, CASP1, CASP3, CASP8, TAP2, TGM3,) were included in a multiple regression model. The results of the analysis indicated that none of the variables analyzed had a detectable effect on the duration of viremia.

3.6. Multivariate analysis of factors associated with persistent infection

To determine the ability of the independent variables qRT-PCR, ELISA, and FFN to predict PRRSV persistence or not-persistence, logistic regression was run separately for each independent variable (AUC). The analyses showed that neither qRT-PCR nor ELISA responses were able to predict PRRSV persistence or not-persistence. However, the neutralizing antibody response, as measured by the FFN assay, correctly predicted 79.8% of the cases (coefficient = -0.01 and $p < 0.001$). To determine the effect of qRT-PCR and ELISA as covariates of the FFN response, a multiple logistic regression stepwise model was performed. The model found no effect of the covariates on the relationship of FFN and persistence ($p > 0.05$) (Table 6).

4. DISCUSSION

4.1. PRRSV detection

Persistence is unquestionably the single most significant epidemiological attribute of PRRSV infection. PRRSV produces an acute infection characterized by viremia, clinical disease with seroconversion, and a chronic, persistent infection in which the virus is present at ever-declining levels in a continuously decreasing percentage of convalescent animals over time (Allende et al 2000; Rowland et al. 2003). This characteristic of the virus allows it perpetuate within herds.

The duration of persistence is uncertain, but it is recognized as extensive: Wills et al. (1997) isolated virus from one of 4 pigs 157 days post-inoculation; Allende et al. (2000) detected infectious virus by bioassay in 2 of 5 pigs at day 150 post-inoculation; Horter et al. (2002) detected infectious PRRS virus in 51 of 59 (84%) animals between 63 and 105 days post-inoculation, including 10 of 11 (91%) of animals at day 105 post-inoculation; Rowland et al. (2003) isolated virus from tonsil and lymph nodes from pigs infected *in utero* up to 132 days after farrowing; Batista et al. (2004) detected virus by PCR in tissue pools (tonsil, superficial inguinal and sternal lymph nodes) from 2 of 10 (20%) at 135 days post inoculation (DPI) and from 49 of 50 (98%) animals euthanized 30-100 DPI.

Table 6 . Summary of the multivariate analysis to evaluate possible effect of the parameters of viremia and immune response factors on the duration of viremia, infectious virus persistence and body weight. (inclusion criteria $p < 0.05$)

	Cox prop/hazard reg[1]		MLR[2]		MR[3]	
	Viremia (Surv)[4] (qRTR-PCR)		Persistence (Bioasay or VI)		Body weight (kg)	
Variables	Exp(b)	95% CI	Coeff.[5]	OR[6]	Coeff.[5]	*t*[7]
Immune response						
ELISA (AUC)						
ELISA (Peak)						
ELISA (Time to peak)						
ELISA (Duration)						
FFN (AUC)			-0.01	0.992	0.349	4.705
FFN (Peak)						
FFN (Time to peak)						
FFN (Duration)						
Viremia						
qRT-PCR (AUC)						
qRT-PCR (Peak)						
qRT-PCR (Time to peak)						
qRT-PCR (Duration)						
Cytokines expression						
IFNG					31.141	2.625
IL12B						
IL15						
IRF1						
CTIIA						
TNF			5.156	17.34	-56.07	-3.409
NOS2A						
CXXL10						
IL13						
IL5						
IL10						
CASP1						
CASP3						
CASP8						
TAP2						
TGM3						
Pig genetic (genotypes)						
SLA-1 (1.0 / 22.0)						
SLA-1(1.0 / 39.0)						
SLA-1(4.0 / 6.0)						

[1] Cox proportional-hazard regression.

[2] Multiple logistic regressions.

[3] Multiple regressions

[4] Survival analyses

[5] Regression coefficients

[6] Odds ratios

[7] *t* test

In this study, the detection of PRRSV in serum showed that all pigs were productively infected with PRRSV. The magnitude of viremia peaked at between 7 and 21 DPI. Thereafter, most pigs cleared the viremia by 42 DPI; but, in small percentage (2 to 7%), virus was detected by qRT-PCR for up to 154 DPI. In contrast, lymphoid tissues (tonsil, submandibular and superficial lymph nodes) were positive by qRT-PCR through 202 DPI in one or more pigs at each sampling point.

In agreement with previous publications (Allende et al 2000; Horter et al., 2002; Rowland et al. 2003; Wills et al., 1997), the duration of infection as determined by virus isolation on serum and lymphoid tissues was of short duration. Based on bioassays, virus continued to replicate in lymphoid tissues for up to 175 DPI. These results suggest that infectious virus is able to persist in populations for a longer period of time than previously thought (Zimmerman 2003).

Comparison of these diagnostic tests showed that qRT-PCR was the most sensitive assay for detecting PRRSV infected pigs, but the discrepancy between PCR and bioassay results suggests that PCR is probably detecting non-infectious virus or the amount of infectious virus was under the analytical sensitivity of the assay. These findings should be taken in account when protocols for eradication or elimination of PRRSV from infected farms.

In addition, paired comparison of qualitative results obtained from the qRT-PCR, ORF5-nPCR and ORF7-nPCR indicated that nested PCR assays were best related to the presence of infectious virus detected by bioassay. These finding suggested that although the commercial qRT-PCR is good test to demonstrate PRRSV viremia, nested PCR focused on ORF5 or ORF7, are more prognostic of persistence

4.2. Body weight

The effect of PRRSV on growth performance in weaning to market pigs has been reported in surveillance studies (Regula et al., 2000) and experimental studies (Greiner et al., 2000; Thacker, 2003) at early stage of the infection. Studies analyzing the effect of PRRSV infection on growth and performance of pig evaluated the effects of dual infections of PRRSV with swine influenza virus (Van Reeth et al., 2001), Mycoplasma hyopneumoniae

(Roberts et al., 2003) or porcine circovirus type 2 (Kritas et al., 2007). Since the attributed participation of PRRSV on post-weaning multi-systemic syndrome (PWMS) (Pogranichny, et al., 2002; Wellenberg et al., 2004), the logical explanation for the presence of reduced-growth pigs ("light" pigs), was the presence of a concurrent PRRSV and porcine circovirus (PCV2) infection, which would be consistent with PWMS. However, in this experiment, all pigs were negative for the presence of porcine circovirus by PCR. In this experiment the effect of PRRSV infection was evaluated for long time after infection. In agreement with Thacker (2003), these results indicated that an uncomplicated PRRSV infection could produce a negative effect of pig growth performance with a significant difference in average daily gain. One possible explanation could be the found in the significant statistical association between the viremia and the presence of "light" pigs.

Another explanation for the presence of poor-doing pigs would include the overproduction of inflammatory mediators such as IL-1β, TNF-α, IL-6 and IL-8, which alter metabolism and feeding behavior of the pigs. Data analysis indicated that genetic expression of interferon (INF) γ and tumor necrosis factor (TNF) were significantly associated with variation on body weight. Greiner (2000) suggested that the magnitude of the reduction in weight gain was dependent on the stage of the virus infection and IFN concentration in response to PRRSV infection.

4.3. Serum antibody detection

PRRSV specific antibodies appear rapidly after infection with serum antibodies detectable by 5-7 days post-infection (DPI) and most animals are seropositive by DPI 14 (Johnson et al., 2004; Labarque et al., 2000; van der Linden et al., 2003; Yoon et al., 1995). Most antibodies are directed to the N protein, the immunodominant epitope (Yoon, et al., 1995), but these antibodies do not confer protection (Labarque et al., 2000, Yoon et al., 1994). PRRSV-specific antibodies are detectable early after infection, with PRRSV-specific immunoglobulin M (IgM) detected in the serum and BAL fluid 9-14 DPI, declining to undetectable levels by 28-42 DPI (Joo et al., 1997; Labarque et al., 2000; Loemba et al., 1996; Park et al., 1995; Vezina et al., 1996). In contrast, IgG levels remain high in serum and BAL fluid for weeks or months (Labarque et al., 2000).

The specific humoral response is against PRRSV has been recognized as problematic. That is, the function of the neutralizing antibodies in the prevention and clearance of the virus infection is contradictory (Osorio et al., 2002; Batista et al., 2004). In particular, the extended coexistence of virus and antibodies in body fluids suggested atypical or ineffective responses in both humoral and cellular components (Allende et al., 2000; Batista et al., 2004; Meng, 2000; van Woensel et al., 1998). This inadequate immune response and the inherent genetic diversity of the virus are factors primarily contributing to PRRSV persistence (Meng, 2000). As reported previously (Batista et al., 2004, Kim et al., 2007, Lopez and Osorio 2004, Yoon et al., 1995) serum neutralizing antibody usually appear ≥ 4 weeks following inoculation. In this study, neutralizing antibodies were first detected on DPI 14, with the peak response at DPI 56 (90 of 97). In agreement with Nelson (1994), a high percent of pigs maintained a detectable neutralizing response for extended period after infection, e.g., at DPI 202, 7 of 9 pigs tested FFN positive.

4.4. PRRSV sequencing

The ORF5 protein (GP5) is considered highly variable and primarily responsible for the diversity of field isolates (Allende et al., 2000; Rowland et al., 1999). In this study, phylogenetic analysis of ORF5 sequences reveled little genetic diversity over time. That is, sequences recovered late in the course of the study were still very closely related both to the the original inoculum (CC-01-04) and to contemporary sequences. The low degree of genetic variation between the PRRSV isolates and the original inoculum suggested that recovered virus from bioassay pigs were not a cross contamination in the handling of samples. In this study, genetic changes on ORF5 sequences did not show a specific pattern associable with the time after inoculation that the viruses were recovered.

4.5. Survival Analysis

The duration of viremia, indicated by the detection of PRRSV RNA using qRT-PCR presented in survival curves (Figure 2), suggested that the duration of the viremia was not different between PRRSV persistent and not-persistent pigs. Moreover, neither antibody response, measured by ELISA or FFN nor cytokine genetic expression (IFNG, IL12B, IL15,

IRF1, CTIIA, TNF, NOS2A, CXXL10, IL13, IL5, IL10, CASP1, CASP3, CASP8, TAP2, TGM3,) had a detectable effect on the duration of viremia. On the other hand, the duration of humoral response, as measured by ELISA, indicated that the median survival between PRRSV persistent and not-persistent group of pigs were significantly different. Similarly, survival curve analysis of the duration of neutralizing antibody response estimated using FFN indicated that PRRSV persistent pigs developed a significant shorter immune response than not persistent pigs. This finding suggested that PRRSV persistent pigs develop shorter immune response than the not persistent pigs, including the development of neutralizing antibodies. Statistical analysis demonstrated that overall response qRT-PCR (virus load) had no effect on the humoral response measured by ELISA or FFN.

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Chapter 3. Evaluation of the risk of PRRSV transmission via ingestion of muscle from persistently-infected pigs

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SUMMARY

The objectives of this experiment were to determine how long porcine reproductive and respiratory syndrome virus (PRRSV) could be detected in muscle tissues of experimentally infected pigs and to evaluate the transmissibility of PRRSV to pigs via ingestion of qRT-PCR-positive muscle tissues. Serum, lymphoid tissues, and muscle (*M. longissimus dorsi*) samples were collected from 135 pigs (89 PRRSV-inoculated pigs and 46 negative control). Between 28 and 202 days post inoculation (DPI), 13 of 89 (14.6%) muscle samples were positive by qRT-PCR. Among these 13, PRRSV was isolated from 4 of the 13 corresponding serum samples and 3 of 13 lymphoid tissue samples. In addition, infectious virus in lymphoid tissue homogenates of 6 of 13 pigs by intramuscular bioassay. Swine transmissibility studies were performed by feeding thirteen 3-week-old PRRSV-naive pigs (recipient pigs) qRT-PCR-positive muscle and then monitoring recipients for evidence of PRRSV viremia by qRT-PCR. No transmission of PRRSV to recipient pigs via consumption of muscle samples was observed. These data suggested that qRT-PCR detected non-infectious PRRSV in pig meat and/or PRRSV is not highly transmissible to susceptible pigs via consumption of PRRSV-contaminated meat.

INTRODUCTION

First identified in 1991 (Wensvoort et al., 1991), porcine reproductive and respiratory syndrome virus (PRRSV) has since become endemic in most areas of swine production in the world [Newman et al., 2005; Zimmerman, 2003]. The possible introduction of the virus into

PRRSV-free countries via the import of pig meat became a trade issue early in the pandemic. Bloemraad et al. (1994) first reported that virus was present in muscle tissue collected from viremic pigs, albeit at low virus titers, and that the virus was only slightly affected by storage for up to 48 hour at 4ºC (39ºF). Under experimental conditions, van der Linded et al. (2003) reported that PRRSV "could be infectious through the oral route via the feeding of meat obtained from recently infected pigs." In the field, Margar and Larochelle (2004) reported low levels of PRRSV in a small percentage of pig meat collected at an abattoir. When fed raw PRRSV-contaminated pig meat under experimental conditions, some pigs became infected. Several risk analyses have been conducted to evaluate the probability of introducing PRRSV through the import of pig meat from PRRSV-infected countries (Anonymous, 2005; Banks et al., 2004; Garner et al., 2001; Pharo, 2006). Although they have important implications for international commercial trade, these analyses are based on very sparse data. Therefore, the objectives of this experiment were to determine how long PRRSV could be detected in the muscle tissues and other tissues of experimentally infected pigs and investigate the potential of oral PRRSV transmission to naïve pigs via muscle samples collected from donor pigs of known disease status and temporal stage of infection.

MATERIALS AND METHODS

Experimental design

The objectives of this experiment were to determine how long PRRSV could be detected in the muscle tissues of experimentally infected pigs (Trials 1 and 2) and evaluate the transmissibility of PRRSV-positive muscle tissue to pigs via ingestion (Trial 3). In Trial 1, tissues and serum were collected over time from donor pigs exposed to PRRSV under experimental conditions. Serum, lymphoid tissues, and muscle transudates were assayed for PRRSV by qRT-PCR, virus isolation (VI) and/or intramuscular inoculation of swine (Trial 2). In Trial 3, qRT-PCR-positive muscle samples were tested for infectivity by feeding to PRRSV-naïve young pigs.

Porcine reproductive and respiratory syndrome virus

Virus strain

The North American prototype PRRSV, ATCC VR-2332 (American Type Culture Collection, Manassas, VA, USA) was used in this study. The complete genomic sequence for VR-2332 has been published (GenBank® accession number PRU87392). The virus used in this study was derived from a plaque-cloned virus (CC-01) described by Chang et al., 2002. Relative to VR-2332, the ORF 5 nucleotide sequence of CC-01 differed at three positions: 38 (G to A), 252 (C to T), and 451 (A to G). These changes resulted in amino acid substitutions at two residues: 13 (arginine to glutamine) and 151 (arginine to glycine).

Virus propagation

The inoculum for this experiment was prepared by infecting a 21 day-old pig with PRRSV isolate CC-01. Serum collected 7 days post inoculation (DPI) was used to infect cell cultures followed by two additional passages to produce a sufficient volume of inoculum for the experiment. Virus was propagated on 24 hr-old confluent MARC-145 cells (Kim et al., 1993). Cells were prepared in 162 cm^2 flasks (Corning Inc., Corning, NY, USA) containing growth medium: MEM (Sigma Chemical Co., St. Louis, MO USA) supplemented with 10% fetal bovine serum (FBS), 50 µg per ml gentamicin, 100 IU per ml penicillin, 100 mg per ml streptomycin, and 0.25 µg per ml amphotericin B (Sigma Chemical). After 24 hr at 37°C in a humidified 5% CO_2 incubator, the MEM growth medium was discarded and the flasks inoculated with 5 ml of serum. After 2 hr at 37°C in a humidified 5% CO_2 incubator, the inoculum was discarded and 50 ml of maintenance medium was added: 50 ml of MEM supplemented with 5% FBS, 50 µg per ml gentamicin, 100 IU per ml penicillin, 100 µg per ml streptomycin, and 0.25 µg per ml amphotericin B (Sigma Chemical). Thereafter, cells were examined daily for cytopathic effect (CPE). When 75% CPE was observed, the medium was freeze-thawed (-80°C / 25°C) and cell lysates were harvested and stored at -80°C.

Virus titration

Virus titrations were done on MARC-145 cells in 96 well plates (Corning Inc.) containing 200 µl of 4×10^5 cells/ml suspended in MEM growth medium (Sigma Chemical). Ten-fold serial dilutions of stock virus were performed in MEM and then 4 wells were inoculated with 100 µl of each virus dilution. Subsequently, plates were incubated at 37°C and 5% CO_2 for 2 hours; thereafter, the inoculum was discarded and 200 µl of maintenance medium was added to each well. After incubation for 48 hr at 37°C and 5% CO_2, medium was discarded, cells were washed with PBS, fixed with aqueous 80% acetone solution, and stained with fluorescein isothiocyanate-conjugated monoclonal antibody SDOW17 (Rural Technologies Inc., Brookings, South Dakota, USA).

Animal care and handling

Animal care and animal handling procedures were approved by the Biosafety Committee and the Animal Care and Use Committee at Iowa State University. The project was conducted in Iowa State University facilities in compliance with guidelines outlined in the *International Guiding Principles for Biomedical Research Involving Animals* as issued by the *Council for the International Organizations of Medical Sciences*. For the three trials described herein, pigs were received into Iowa State University facilities and observed for at least one week prior to the commencement of experimental procedures. PRRSV-negative status was confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) on serum samples collected upon arrival and on day zero of the experiment.

Trial 1: PRRSV donor pigs.

Donor pigs were used to provide tissue samples for bioassays conducted in Trial 2 and Trial 3. Fourteen-day-old (n = 165) PRRSV-negative large white/landrace cross piglets (50% barrows and 50% gilts) were received at the Iowa State University Livestock Infectious Disease Isolation Facility one week prior to the beginning of the experiment and randomly assigned to treatments by blindly selecting ear tags (pigs were ear-tagged upon arrival). Tag numbers 1–109 (n = 109) were assigned to the PRRSV-inoculated group; numbers 110–165 (n = 56) were assigned to the negative control group. Pigs were divided

equally by gender between the infected and control groups. Even-numbered tags were assigned to males and odd-numbered tags were assigned to females.

Among the 165 animals, 30 pigs were excluded from the experiment because they were too small (<42 days of age) to provide muscle samples of a size adequate for the oral transmission trial, died unexpectedly, or were in a group of pigs that was re-challenged with PRRSV on DPI 193. Therefore, serum, lymphoid tissues, and muscle samples were available from a subset of 135 pigs consisting of 89 PRRSV donor pigs and 46 negative control pigs. Donor pigs (n = 109) were intramuscularly (IM) administered one ml of PRRSV ($1 \times 10^{4.0}$ $TCID_{50}$) diluted in minimum essential medium (MEM) (Sigma Chemical Co., St. Louis, MO USA). Negative control pigs (n = 56) were sham inoculated with MEM by IM.

Over the course of the observation period, animals were euthanized at ~14 day intervals in declining sequential order (i.e., larger to smaller), with the last pigs euthanized at 202 days post inoculation (DPI). Five pigs (3 PRRSV-inoculated and2 negative control) were euthanized at each sampling point from DPI 28 through DPI 112; thereafter, approximately 15 pigs (10 PRRSV-inoculated; 5 negative control) were euthanized per sampling. Serum, lymphoid tissue (tonsil, superficial inguinal and submandibular lymph nodes) and *M. longissimus dorsi* samples were collected from each pig after euthanasia.

Trial 2: Intramuscular bioassay pigs

Intramuscular (IM) bioassay pigs were used to screen tissue homogenates for infectious PRRSV. To preclude transmission among animals, IM bioassay pigs were individually housed under biosafety level 2 conditions. Each bioassay pig was IM inoculated with clarified, but not filtered, homogenate prepared from tonsil (7 ml), submandibular (7 ml) and superficial inguinal (7 ml) lymph nodes collected at necropsy from one donor pig. Preparation of tissue homogenates is described below (see *Trial 1: tissue collection and processing)*. Following inoculation, pigs were monitored for evidence PRRSV infection over a two-week period by assaying serum samples collected on 0, 7 and 14 DPI using PRRSV qRT-PCR.

Trial 3: Oral bioassay pigs

The oral transmission experiment was conducted to evaluate the infectivity of qRT-PCR-positive muscle tissue for young pigs. To preclude transmission among animals, pigs were individually-housed in hepa-filtered (Flanders Filters, Inc., Washington, NC, USA) isolation units (Barrier Systems, Inc., Toms River, NJ, USA). Prior to the commencement of the transmission experiment, pigs were trained to ingest raw pig muscle for four days by feeding 100g of muscle from a PRRSV-free pig mixed with sweetened condensed milk. On the day of exposure, pigs were deprived of food (not water) for 12 hours. Each oral bioassay pig (n= 13) was then fed 100 to 200 grams of unprocessed muscle (*M. longissimus dorsi*) collected from the right side of the 13 pigs with qRT-PCR-positive muscle samples. Thereafter, oral bioassay pigs were monitored for evidence of PRRSV infection by testing serum samples collected on days 7 and 14 post-feeding using qRT-PCR.

Sample collection and processing

Serum collection

Blood samples were collected from pigs using a single-use blood collection system (Kendall Company, Manfield, MA, USA), then centrifuged at 1000 × *g* for 10 min. Serum was harvested, aliquoted into 2.0 ml cryovials (Fisher Scientific Co.,Hanover Park, IL, USA), and stored at -80°C until tested.

Euthanasia protocol

The euthanasia procedure was performed in two steps. First, pigs received an intramuscular administration (0.025 ml per kg of body weight) of a solution formulated by reconstituting Telazol (250 mg of tiletamine, 250 mg of zolazepam; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) with 2.5 ml of xylazine (100mg/ml; Lloyd Laboratories, Shenandoah, Iowa, USA) and 2.5 ml of ketamine (100 mg/ml; Fort Dodge Animal Health). When recumbent and sedated, the pigs received an intravenous administration (1.0 ml per kg of body weight) of a solution containing butorphanol (0.2 mg per ml; Fort Dodge Animal Health), xylazine (2.0 mg per ml; Lloyd Laboratories), and ketamine (2.0 mg per ml; Fort Dodge Animal Health). When pigs achieved a surgical plan of anaesthesia they were

exsanguinated.

Trial 1: tissue collection and processing

At necropsy, two sections (left and right sides) of muscle (*M. longissimus dorsi*) were collected from the lumbar area. Thereafter, lymphoid tissue samples (tonsil, superficial inguinal and submandibular lymph nodes) were collected and dissected. One section of each tissue was deposited in a sterile 4.0 ml tube (Fisher Scientific Co.); the remainder was placed in a sterile plastic bag (NASCO, Fort Atkinson, WI, USA). Specific measures were taken to eliminate virus cross-contamination at necropsy: 1) negative control animals were necropsied before PRRSV-inoculated pigs; 2) carcasses were washed with a disinfectant solution (Pharmacal Research Labs Inc., Naugatuck, CT, USA); 3) necropsy instruments were washed, immersed in methanol, and flamed between each tissue specimen collection; 4) specimens were directly placed in sterile plastic bags (NASCO); 5) specimens were placed on wet ice immediately after collection; 6) latex gloves (Cardinal Health, McGaw Park, IL, USA) were changed between each pig. All samples were stored at -80°C until tested.

Muscle transudates from the left side of each Trial 1 pig were assayed by qRT-PCR to identify virus-positive muscle tissues. The corresponding "right side" muscle samples were maintained frozen at -21°C for use in the oral transmission experiment (Trial 3).

Muscle transudate was collected using two different processes in order to determine if one provided a greater harvest of virus. To preserve virus infectivity, the muscle sample from the left side of each donor pig was divided in two while still frozen and each half processed separately. One half was minced in a sterile muscle grinder (LEM Products Inc. Harrison OH, USA) while still frozen and placed in a plastic bag. The second (intact) half was placed in a sterile plastic bag (SC Johnson & Son Inc. Racine, WI, USA). Both intact and ground muscle samples were held at 4°C for 8 to 12 hr, during which time the transudate pooled in the plastic bag. The transudate was decanted into a 50 ml centrifuge tube (Fisher Scientific) and the pH measured. Thereafter, the samples were clarified (1000 × *g* for 10 min), aliquoted into 2 ml snap-cap tubes (Fisher Scientific), and stored at -80°C until assayed. Each muscle transudate sample was tested by qRT-PCR, i.e., each pig was tested in

duplicate.

Tissue homogenates from Trial 1 pigs were tested for infectious PRRSV by bioassay (Trial 2). Homogenates were prepared separately for each specimen (tonsil, submandibular lymph node, superficial inguinal lymph node) by mincing 2 grams of tissue and then mixing with 8 ml of MEM (Sigma Chemical) supplemented with 5% FBS (Sigma Chemical) without antibiotics. The preparation was then macerated (Stomacher®80 Biomaster, Fisher Scientific) and centrifuged (4,500 × *g*) for 20 min. Following centrifugation, supernatant was collected and immediately administered to bioassay pigs (Trial 2).

Diagnostic assays

PRRSV ELISA

Serum samples were tested for PRRS virus-specific antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) kit (HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Westbrook, ME, USA).

Virus isolation

Virus isolation was performed following the virus propagation protocol described above except that serum or tissue homogenate samples were inoculated onto 4 replicate wells of a 96-well plate, after which plates were incubated at 37°C in a humidified 5% CO_2 incubator for one hour. Thereafter, the inoculum was discarded and 200 µl of maintenance medium was added to each well. Plates were incubated for an additional 5 days at 37°C in a humidified 5% CO_2 incubator. The presence of virus was determined by microscopic observation for typical cytopathic effects. Subsequently, cells were washed with PBS, fixed with 80% acetone solution, and stained with fluorescein isothiocyanate-conjugated monoclonal antibody FITC-SDOW-17 (Rural Technologies Inc., Brookings, South Dakota).

PRRSV reverse-transcriptase polymerase chain reaction

Quantitative, real-time RT-PCR was conducted as previously described using a commercially available kit (Tetracore Inc., Rockville, MD, USA) (Wasilk et al, 2004). In brief, RNA extraction from serum and muscle tissue fluids was performed using the QIAamp Viral RNA Mini-Kit® (Qiagen Inc., Valencia, CA, USA) following the kit instructions. For

RNA extraction from tissues, approximately one gram of tissue was mixed and homogenized with an equal volume of phosphate buffer solution (PBS), then a quanidinium thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine) was added and RNA was obtained through the Qiagen Qiashredder columns and Rneasy Mini kit. Quantification of samples was expressed in number of RNA copies per ml for fluids and copies per gram for solid tissues using linear extrapolation of the cycle threshold values against a standard curve generated by serially dilutions of known amounts of *in vitro* transcript RNA product (1×10^{-1} to 1×10^{8}copies/µl).

Statistical analysis

Initially, results were summarized using descriptive statistics. Thereafter, qRT-PCR results on ground and unprocessed muscle samples were compared by paired Student's *t* test. PRRSV RNA copies per ml detected by qRT-PCR on serum, muscle and the means of qRT-PCR results on individual lymphoid tissue samples (tonsil, submandibular lymph nodes and inguinal lymph nodes) were evaluated using Spearman's coefficient rank correlation. Finally, the proportions of PRRSV-positive samples detected by qRT-PCR, virus isolation, and IM bioassay were compared using McNemar's exact probability test. Analyses were performed using MedCalc® 9.2.1.0 (MedCalc Software, Mariakerke Belgium).

RESULTS

Trial 1: PRRSV donor pigs

In Trial 1, PRRSV-positive muscle tissues were identified over time post-exposure in 89 experimentally-inoculated pigs. Forty-six pigs served as age-matched controls. All pigs were determined to be free of PRRSV infection by qRT-PCR analysis of serum samples collected upon arrival into the animal holding facilities at DPI -7 and again on DPI 0. Following exposure to PRRSV, all inoculated pigs were confirmed to be infected on the basis of qRT-PCR-positive results on serum samples collected on DPI 7 and 14. All negative control animals remained free of PRRSV infection throughout the course of the experiment.

PRRSV-positive muscle tissues were identified by qRT-PCR testing of muscle transudate. Both ground and intact muscle transudates were tested to compare the effect of

meat processing on virus detection. As a result, each pig was tested twice. Muscle tissue samples from 13 of 89 pigs euthanized between 28 and 202 DPI tested positive for PRRSV RNA (Table 1). Four of the 13 muscle specimens were qRT-PCR-positive on both intact and ground muscle transudates, 5 were positive on intact muscle transudate only, and 4 were positive on transudate from ground muscle. Based on qRT-PCR results, the mean PRRSV copies per ml was $1 \times 10^{3.7}$ in transudate from ground meat (n = 8) and $1 \times 10^{3.8}$ in intact meat (n = 9). Given the sparse and highly correlated data, no further statistical comparisons of the effect of muscle tissue processing on virus detection in transudate were possible. Hereafter, in the 4 cases in which both intact and ground muscle transudates were positive, the means of the qRT-PCR results were used in statistical comparisons.

By time post-inoculation, qRT-PCR-positive muscle transudate was detected in 3 of 3 pigs euthanized on DPI 28, 2 of 3 pigs on DPIs 42, 56, and 70; and 1 of 3 pigs on DPI 84. Thereafter, PRRSV was detected in muscle transudate on DPIs 147 (1 of 10 pigs), 161(1 of 10 pigs) and 202 (1 of 9 pigs). As shown in Table 1, 12 of 13 muscle tissue qRT-PCR-positive pigs were also qRT-PCR positive on lymphoid tissues (McNemar, $p = 1.0$). In contrast, 6 of the 13 pigs were serum qRT-PCR positive for PRRSV (McNemar, $p = 0.02$). Analysis of the qRT-PCR results found no correlation in PRRSV copies per ml between serum versus muscle (rho = 0.46), serum versus lymphoid tissue (rho = 0.52), or muscle versus lymphoid tissue (rho = 0.08) using Spearman's coefficient rank correlation.

Virus isolation was also attempted on the identical samples that were assayed by qRT-PCR. PRRSV was recovered from serum (n = 4) and lymphoid tissue (n = 3) samples prior to DPI 56 (Table 1). No virus was isolated from muscle tissue transudate. With the exception of one serum sample (DPI 56), the samples from which virus was isolated were also positive by qRT-PCR, but 3 qRT-PCR positive serum samples were VI negative.

Table 1. PRRSV detection: Summary of Trials 1, 2, and 3.

		Trial 1 [a]							Trial 2	Trial 3
		PRRSV qRT-PCR				PRRSV Virus Isolation			Swine (IM)	Swine (Oral)
DPI [b]	Pig	Muscle [c]	Muscle [d]	Serum	LT [e]	Muscle	Serum	LT	LT	Muscle
28	106	$1\times10^{4.6}$	$1\times10^{5.4}$	$1\times10^{4.9}$	$1\times10^{6.0}$	neg	POS	POS	POS	neg
28	105	$1\times10^{4.6}$	$1\times10^{5.2}$	$1\times10^{4.9}$	$1\times10^{5.4}$	neg	POS	POS	POS	neg
28	104	$1\times10^{4.8}$	neg	$1\times10^{3.9}$	$1\times10^{5.5}$	neg	POS	POS	POS	neg
42	103	neg	$1\times10^{2.9}$	neg	$1\times10^{5.4}$	neg	neg	neg	neg	neg
42	101	$1\times10^{3.4}$	neg	$1\times10^{4.2}$	$1\times10^{6.0}$	neg	neg	neg	neg	neg
56	99	$1\times10^{3.7}$	neg	neg	$1\times10^{6.6}$	neg	neg	neg	POS	neg
56	98	$1\times10^{2.1}$	$1\times10^{3.8}$	neg	$1\times10^{5.3}$	neg	POS	neg	POS	neg
70	97	$1\times10^{2.4}$	neg	$1\times10^{2.2}$	$1\times10^{6.1}$	neg	neg	neg	neg	neg
70	96	$1\times10^{2.9}$	$1\times10^{2.7}$	$1\times10^{2.5}$	$1\times10^{4.5}$	neg	neg	neg	POS	neg
84	94	neg	$1\times10^{4.2}$	neg	$1\times10^{4.0}$	neg	neg	neg	neg	neg
147	57	$1\times10^{5.0}$	neg	neg	$1\times10^{3.1}$	neg	neg	neg	neg	neg
161	52	neg	$1\times10^{2.5}$	neg	$1\times10^{2.4}$	neg	neg	neg	neg	neg
202	8	neg	$1\times10^{3.1}$	neg	neg	neg	neg	neg	neg	neg

[a] All Trial 1 negative control animals were negative for PRRSV by qRT-PCR, VI, and swine (IM) bioassay

[b] Day post-inoculation

[c] Intact muscle (RNA copies per ml)

[d] Ground muscle (RNA copies per ml)

[e] Lymphoid tissue (RNA copies per gram). Mean qRT-PCR results on tonsil, submandibular, and inguinal lymph nodes

Overall, qRT-PCR testing on samples collected after 28 DPI found that 7 of 89 (7.7%) serum samples, 62 of 89 (69.6%) lymphoid tissues samples, and 13 of 89 (14.6%) muscle samples were positive. Infectious virus was recovered from 4 of 13 serum samples and 3 of 13 lymphoid tissue samples by virus isolation; 6 of 13 pigs were positive by bioassay (Table 1). Attempts at recovering infectious PRRSV from meat transudate samples were unsuccessful.

Trial 2: Intramuscular bioassay pigs

Trial 2 (IM bioassays) were conducted by inoculating pigs with lymphoid tissue homogenate prepared from tonsil, submandibular lymph node, and superficial inguinal lymph node recovered from the 13 muscle tissue-positive pigs identified in Trial 1. The presence of infectious PRRSV in tissue homogenate was indicated by the detection of PRRSV viremia in bioassay pigs following exposure. Infectious PRRSV was detected by IM bioassay of lymphoid tissues in 6 of the 13 Trial 1 pigs, including one pig euthanized at 70 DPI (Table 1). As shown in Table 1, there was significant disagreement between the proportion of pigs that were shown to be infected with PRRSV by IM bioassay positive in Trial 2 (6 of 13) and the proportion of pigs in Trial 1 that were qRT-PCR-positive on lymphoid tissues (12 of 13, McNemar, $p = 0.03$) or muscle transudate (13 of 13, McNemar, $p = 0.02$). In contrast, there was significant agreement between the proportions of pig that were IM bioassay positive in Trial 2 and Trial 1 pigs that were virus isolation positive on serum (4 of 13, McNemar, $p = 0.69$) or lymphoid tissues (3 of 13, McNemar, $p = 0.50$).

Trial 3: Oral bioassay pigs

Evaluation of the transmissibility of virus by oral intake of qRT-PCR-positive muscle samples was performed by feeding qRT-PCR-positive muscle tissue to 4-week old pigs (Trial 3). These pigs had been adapted to consumption of raw meat prior to the experiment; therefore, all Trial 3 pigs consumed the PRRSV-positive muscle sample in less than 5 minutes. The detection of PRRSV viremia was used as the criterion for transmission via PRRSV-positive muscle. Results from qRT-PCR tests on serum samples collected from

Trial 3 pigs on DPI 0, 7, and 14 showed that pigs were PRRSV-negative at the time of exposure and remained negative thereafter (Table 1).

DISCUSSION

Published research on the presence of PRRSV in pig meat includes studies based on samples collected at slaughter and studies conducted under experimental conditions. Early in the PRRSV pandemic, Frey et al. (1995) reported isolation of PRRSV from pooled samples of fresh pig meat collected at slaughter (6 of 1049). On the other hand, Larochelle and Magar (1997) reported no detection of PRRSV by either RT- PCR or virus isolation from tissue homogenates prepared from 438 packages of frozen meat. Likewise, Wang (1999) reported RT-PCR-negative results on muscle tissue samples collected from 472 carcasses. Among this group of animals, 11 of 140 (7.9%) serum samples were RT-PCR positive and 205 of 240 (85.4%) serum samples were positive for PRRSV serum antibodies by ELISA. More recently, Magar et al. (2004) evaluated serum and meat samples collected at slaughter. Among 1039 serum samples, 772 (74.3%) were PRRSV ELISA positive and 45 (4.3%) were RT-PCR positive. Among 1027 meat samples, 19 were RT-PCR positive and PRRSV was isolated from 1 of the 19.

Under experimental conditions, Bloemraad et al. (1994) inoculated 6-month-old pigs with Lelystad virus and isolated PRRSV from muscle specimens at 5 DPI (2 of 2 pigs) and 10 DPI (1 of 2 pigs). Magar et al. (1995) inoculated 6-month-old pigs with Lelystad virus and isolated PRRSV from muscle specimens collected at 5 DPI (2 of 2 pigs), but not at 14 DPI (0 of 2 pigs). Mengeling et al. (1995) inoculated 21 6-week-old pigs with one of 3 PRRSV isolates (VR 2332, NADC-9, Hesse) and isolated PRRSV VR2332 from a muscle sample collected from one pig at 7 DPI. Similarly, van der Linden et al. (2003) inoculated 6-week-old pigs with PRRSV isolates LV ter Huurne or SDSU-73 and isolated PRRSV from approximately 50% of fresh muscle specimens at 11 DPI. Most recently, Cano et al. (2007) inoculated five 45 kg pigs with the PRRSV strain MN-184 and recovered PRRSV from muscle samples collected at 7 DPI using IM swine bioassay. In general, the detection of PRRSV RNA in meat samples of experimentally infected viremic pigs has been reported, but only during the early phase of infection. In this experiment, the presence of viral RNA was

detected in 13 of 89 inoculated pigs for a longer period than previously reported. However, infectious PRRSV was not detected in muscle or muscle transudate samples from these 13 positive detected pigs.

Transmission via the ingestion of muscle from PRRSV-infected pigs has been examined in only 2 studies. Magar et al. (2004) fed 11 pairs of pigs 11 PCR-positive meat samples collected at slaughter. Each pair of pigs was fed 250 to 400 g of meat for 2 consecutive days. Among the 11 pairs of recipient pigs, 7 pairs became infected with PRRSV. van der Linden, et al. (2003) fed meat from PRRSV-inoculated pigs euthanized at 11 DPI to 4 group-housed pigs composed of 12 pigs each. Animals were fed approximately 250 grams per day for 2 days. Transmission was demonstrated in each group, but because the animals were housed in groups, the researchers were unable to identify the meat samples that resulted in transmission as opposed to transmission by pig-to-pig contact within groups.

The intent of this experiment was to expand the current level of information regarding the possible role of meat in PRRSV transmission. Specifically, to determine how long infectious PRRSV persisted in muscle tissue over time post inoculation (Trials 1 and 2) and evaluate the transmissibility of PRRSV-positive muscle tissue for susceptible pigs via ingestion (Trial 3). Efforts to improve the rate of detection by processing (grinding) muscle samples were unsuccessful. That is, although the mean PRRSV RNA copies per ml in transudate from ground meat was slightly higher than the mean PRRSV RNA copies per ml in transudate from intact meat, the means were not significantly different. Thus, these data provide no justification for processing (mincing, grinding) meat samples.

Trial 1 showed that detection of PRRSV in muscle transudate was highly time-dependent (Table 1). That is, PRRSV was detected by qRT-PCR in muscle tissue samples from 9 of 12 pigs euthanized between DPI 28 to 70, but only 4 of 77 pigs euthanized between DPI 84 to 202, i.e., one pig each at DPIs 84, 147, 161, and 202. These data suggest that that PRRSV is more likely to be detected in meat the closer in time the meat is harvested with respect to when the pig was infected. These data, in combination with the observations made in previous publications, suggest that the detection of PRRSV in muscle tissue samples collected at slaughter represents recent infections in finishing pigs as they approach market weight. This interpretation is compatible with the observation made by Magar et al. (2004)

that some RT-PCR-positive meat samples collected at slaughter came from PRRSV antibody-negative pigs, i.e., animals had not had sufficient time to respond immunologically to the infection.

Infectious PRRSV was isolated from serum and/or lymphoid tissue samples from 4 of the 13 pigs with qRT-PCR-positive muscle tissues. Virus isolation was only successful prior to DPI 56. Pig bioassays, i.e., intramuscular inoculation of pigs with lymphoid tissue homogenate (Trial 2), increased the detection of infectious virus to 6 of 13 pigs and extended it to DPI 70. The proportion of pigs in which PRRSV was detected in muscle tissue by qRT-PCR was significantly different (greater) than the proportion in which infectious PRRSV was detected by either virus isolation or IM bioassay (McNemar, $p = 0.016$). Based on these data and similar data from the published reports (Magar et al., 2004; van der Linden et al., 2003), it may be concluded that estimates based upon RT-PCR results will over estimate the prevalence of pig meat containing infectious PRRSV. This conclusion may be contentious because of the common assumption that non-infectious RNA viruses degrade rapidly, but recent reports document the stability of non-infectious, RT-PCR-detectable PRRSV (Baker et al., 2007; Hermann et al, 2007; van der Linden et al., 2003).

To evaluate the transmissibility of PRRSV-positive muscle tissue, 13 pigs were fed qRT-PCR-positive muscle samples (Trial 3). Of the 13 pigs, none became infected, as determined by qRT-PCR analysis of serum samples collected 7 and 14 days after feeding. These results are in contrast to the results of PRRSV transmission via feeding pig meat reported by Magar et al. (2004) and van der Linden et al. (2003). When comparing the three studies, differences in experimental design that could have affected the outcome include: concentration of virus in meat, quantity and number of times pigs were fed PRRSV-contaminated meat, the number of pigs that were fed on from one carcass, and animal housing. Both Magar et al. (2004) and van der Linden et al. (2003) fed greater amounts of PRRSV-contaminated meat (250 to 400 g) and enhanced the likelihood of transmission by feeding 2 pigs per sample for 2 consecutive days. In the case of van der Linden, et al. (2003) animals were housed in groups of 12 pigs each, making interpretation of the transmission data difficult.

Ultimately, risk analyses dealing with the likelihood of PRRSV transmission via ingestion balance on the judgement that extremely rare events may (or may not) occur - events for which probability estimates are generally unavailable. The reports in the literature suggest that infectious PRRSV may be present at low levels in pig meat collected at slaughter, but estimates of the frequency at which pig meat contains PRRSV is highly dependent upon on the assay used to detect the virus, e.g., VI, PCR, or bioassay. Generally considered a highly sensitive assay, the 3 studies that performed RT-PCR on meat samples (Larochelle and Magar, 1997; Magar et al., 2004; Wang, 1999) detected a total of 19 RT-PCR-positive samples among 1937 samples tested (0.98%). However, interpreting these results in terms of the risk of PRRSV transmission via pig meat is complicated by the fact that a positive RT-PCR result does not necessarily mean either that pig meat contains infectious virus or that the virus can be transmitted to susceptible pigs via ingestion. More than anything else, it is this diagnostic ambiguity that complicates risk analyses on PRRSV transmission via the international trade of pork. Thus, the specific elements needed to understand the risk of PRRSV transmission via pig meat include improved methods to differentiate infectious from non-infectious PRRSV and better estimates of the probability of transmission by ingestion (dose: response).

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Chapter 4. Immune response against porcine reproductive and respiratory syndrome virus during acute and chronic infection

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ABSTRACT

A significant obstacle to the prevention and control of porcine reproductive and respiratory syndrome virus (PRRSV) is the inability of current diagnostic tests to provide information concerning the stage of PRRSV infection. To explore possible prognostic combinations of cell-mediated and humoral immune responses, 3-week-old pigs (n = 10) were intramuscularly (IM) inoculated with PRRSV isolate VR-2332 and followed for 193 days post inoculation (DPI). Negative control pigs (n = 10) were IM inoculated with minimum essential medium (MEM). At ~2-week intervals, blood samples were collected from all animals and tested for the number of interferon (IFN)-γ-secreting peripheral blood mononuclear cells (Elispot), PRRSV viremia (qRT-PCR), and serum antibodies using PRRSV protein ELISAs (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) and a commercial PRRSV ELISA (IDEXX Laboratories, Inc.). All pigs were viremic by 7 days post inoculation (DPI), with 50% of the pigs resolving viremia by 56 DPI. A PRRSV-specific IFN-γ response was detected at DPI 28, reached a plateau at 42 DPI, declined slightly, and remained relatively stable from 56 to 193 DPI. On the basis of ROC area under the curve (AUC) analysis, the ELISAs that most reliably differentiated PRRSV-inoculated pigs from negative control pigs were the commercial ELISA (AUC = 0.97), the N ELISA (AUC = 0.96), and the M 3' ELISA (AUC = 0.93). Multivariate analyses were performed to evaluate the relationship between the immune response and the duration and level of viremia. With all antibody assays and Elispot included in the models, the analysis determined that the serum-virus neutralizing antibody response was the best predictor of both level and duration

of viremia. It was concluded that humoral antibody responses, particularly the commercial ELISA, N ELISA, and M 3' ELISA were good predictors of prior exposure to PRRSV, but provided little information regarding the ontogeny of the protective immune response. Likewise, cell-mediated immunity based on the number of IFN-γ-secreting lymphocytes was a poor prognosticator of PRRSV infection status.

1. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, enveloped RNA virus (Benfield et al., 1992; Wensvoort et al., 1991) in genus *Arterivirus* of the family *Arteriviridae* in the order *Nidovirales* (Gorbalenya et al., 2006). PRRSV infection in commercial swine herds leads to reproductive losses in breeding herds and increased morbidity, mortality, and reduced productivity in growing pigs (Zimmerman et al., 2006). Prevention and control of PRRSV has been difficult and direct losses due to PRRSV infection exceed $560 million annually in the U.S. swine herd (Neumann et al., 2005).

The course of PRRSV infection can be divided into three stages: (1) acute infection characterized by systemic infection centered in the lung and lymphoid tissues, viremia, and seroconversion; (2) chronic persistent infection characterized by declining antibody titers and declining levels of virus replication in lymphoid tissues; and (3) clearance of infectious virus by immune mechanisms that are, as yet, poorly described. The majority of animals are able to resolve the PRRSV viremia prior to 60 days post infection (DPI), but the period of post-viremic persistence is uncertain. Horter et al. (2002) detected infectious PRRSV in 51 of 59 (84%) animals between 63 and 105 days post-inoculation, including 10 of 11 (91%) of animals at day 105 post-inoculation. Rowland et al. (2003) isolated virus from tonsil and lymph nodes from pigs infected *in utero* for up to 132 days after farrowing. Wills et al. (1997) isolated virus from one of 4 pigs 157 days post-inoculation and Allende et al. (2000) detected infectious virus by bioassay in 2 of 5 pigs at day 150 post-inoculation.

One of the most significant obstacles to the prevention and control of PRRS is the inability of current tests to determine the stage of PRRSV infection. Ideally, the stage of infection could be characterized by a unique pattern of antibody response against structural and/or non-structural viral proteins, i.e., a prognostic and diagnostic indicator of a pig's

immunity and infectiousness. To test this concept, cell-mediated and humoral immune responses of PRRSV-infected pigs were evaluated over time after inoculation. In addition, the diagnostic performance of a commercial PRRSV enzyme-linked immunosorbent assay (ELISA) and recombinant protein ELISAs (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) were evaluated and compared.

2. MATERIALS AND METHODS

2.1. Experimental Design

The experiment was designed as a longitudinal study of the immune response against PRRSV infection for 193 days post inoculation (DPI). One week prior to the beginning of the experiment, 14-day-old PRRSV-negative Large white/Landrace cross piglets (50% barrows and 50% gilts) were received at the Iowa State University Livestock Infectious Disease Isolation facility. Upon arrival, pigs were randomized to treatments and then serologically tested and determined to be negative for PRRSV infection using a commercial ELISA (HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA) and a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay. On day zero, pigs (n = 10) were intramuscularly (IM) inoculated with one ml of PRRSV ($1 \times 10^{4.0}$ $TCID_{50}$) diluted in minimum essential medium (MEM: Sigma Chemical Co., M4655, St. Louis, MO USA). Negative control pigs (n = 10) were IM inoculated with MEM in the same fashion. All animals were bled at 7- to 14-day intervals for 193 DPI. Thereafter, blood and serum samples were randomly ordered and analyzed for PRRSV and anti-PRRSV responses over time.

2.2. PRRS virus strain and propagation

The North American prototype PRRSV, ATCC VR-2332 (American Type Culture Collection, Manassas, VA, USA) was used in the study. The complete genomic sequence for VR-2332 (GenBank® accession number PRU87392) has been published (Nelsen et al., 1999). The specific virus isolate used in this experiment was derived from a highly homologous plaque-cloned virus (CC-01) described previously (Chang et al., 2002). For this

experiment, isolate CC-01 was expanded by inoculation into a 21-day-old pig and then propagation on 24 hr-old confluent MARC-145 cells using serum collected at 7 DPI. MARC-145 cells were prepared in 162 cm^2 flasks (Costar, 3150, Corning Inc. Corning, NY, USA) containing MEM growth medium: MEM supplemented with 10% fetal bovine serum (FBS) (Sigma, F4922), 50 µg per ml gentamicin (Sigma, G1272), 100 IU per ml penicillin (Sigma, G6784), 100 µg per ml streptomycin (Sigma, G6784), and 0.25 µg per ml amphotericin B (Sigma, A4888). After 24 hr at 37°C in a humidified 5% CO_2 incubator, the MEM growth medium was discarded and the flasks inoculated with 5 ml of serum. After 2 hr at 37°C in a humidified 5% CO_2 incubator, the inoculum was discarded and 50 ml of maintenance medium was added: 50 ml of MEM supplemented with 5% FBS, 50 µg per ml gentamicin, 100 IU per ml penicillin, 100 µg per ml streptomycin, and 0.25 µg per ml amphotericin B. Thereafter, cells were examined daily for cytopathic effect (CPE). When 75% CPE was observed, the medium was freeze-thawed (-80°C / 25°C) and cell lysates were harvested and stored at -80°C.

2.3. Animal care, handling, and sampling

Experimental design, animal care, and animal handling procedures were approved by the Biosafety and Animal Care and Use Committees at Iowa State University, in compliance with the requirements given in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Federation of Animal Science Societies, 1999).

Serum samples were collected from pigs using a single-use blood collection system (Kendall Company, Manfield, MA, USA). Serum was harvested by centrifuging collection tubes at 1000 × *g* for 10 min. Thereafter, serum was aliquoted into 2.0 ml cryovials (Fisher Scientific Co.,Hanover Park, IL, USA) and stored at -80°C until tested.

To harvest peripheral blood mononuclear cells (PBMCs) for Elispot assays, whole blood was harvested in BD Vacutainer™ CPT™ tubes with sodium citrate (Pharmingen, San Diego, CA, USA), and centrifuged at 1300 × *g* at room temperature for 25 min. Thereafter, two milliliters of PBMC at the interface from each tube, were collected into a 50 ml polypropylene centrifuge tube (BD Biosciences, Franklin Lakes, NJ, USA) containing 45 ml of cold PBS. Tubes were centrifuged at 220 × *g*, 4 °C for 15 min. Supernatant was

discarded, the pellet resuspended and incubated for 5 min at room temperature and centrifuged at 220 × *g*, 4 °C for 10 min. The pellet was resuspended in 5.5ml of cold RPMI-1640 cell culture media (Sigma) with 10% FBS. The number of cells per ml was calculated using an automatic cell counter (Beckman Coulter, Inc., Fullerton, CA USA). After the final wash cells were resuspended in 3 ml of freezing medium containing 70% RPMI growth media with 10% FBS, 20% FBS, and 10% DMSO, aliquoted into 2.0 ml cryovials (Corning Incorporated, Corning, NY USA), and maintained at -80 until tested.

2.4. PRRSV quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Quantitative, real-time RT-PCR was conducted as previously described (Wasilk et al., 2004) using a commercially available kit (Tetracore Inc., Rockville, MD, USA). In brief, RNA extraction was performed using the QIAamp® Viral RNA Mini-Kit (Qiagen Inc., Valencia, CA, USA) following the kit instructions. For RNA extraction from tissues, approximately one gram of tissue was weighed and homogenized with an equal volume of phosphate buffered saline solution (PBS). A quanidinium thiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine) was added and RNA was obtained through the Qiagen® Qiashredder™ columns and RNeasy® Mini kit. Quantification of samples was expressed in number of RNA copies per ml for fluids and copies per gram for solid tissues using linear extrapolation of the cycle threshold values against a standard curve generated by serially dilutions of known amounts of *in vitro* transcript RNA product (1×10^{-1} to 1×10^{8}copies/μl).

2.5. Serum antibody detection

2.5.1. Serum virus neutralization

PRRSV neutralizing antibody response in serum was measured using a modified fluorescent focus neutralizing assay (FFN). As described by Wu et al. (2001), two-fold dilutions (1:4 to 1:512) of heat-inactivated pig serum samples were prepared on 96-well plates in MEM (GIBCO®, Invitrogen™ Corporation, Carlsbad, CA USA) supplemented with 2% horse serum (Atlanta Biologicals Inc., Lawrenceville, GA USA). An equal volume of the homologous virus (ATCC VR-2332) at concentration of 2 x 10^3 $TCID_{50}$ per ml was

added to each sample, incubated for one hr at 37°C and then transferred to a 96-well plate containing confluent MARC-145 cells. After 24 hr, the plates were washed, fixed in 80% acetone (Fisherbrand®, Fisher Scientific Co., L.L.C., Hanover Park, IL USA) and stained with FITC-conjugated monoclonal antibody (MAb) SDOW17 FITC (South Dakota State University, Brookings, SD USA) diluted 1:100 in PBS with 5% horse serum in PBS. Neutralizing activity was reported as the last dilution that showed 90% or greater reduction in the number of fluorescent foci.

2.5.2 Commercial ELISA

A commercial ELISA (HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA) was used to detect PRRS virus-specific antibody in serum samples. The assay was performed according to the manufacturer's instructions. A sample was considered positive for PRRS virus antibody if the sample-to-positive (S/P) ratio was ≥ 0.4.

2.5.3. Protein-specific antibody ELISAs

ELISA antigens were produced as recombinant proteins expressed in *E. coli* and purified by immobilized metal affinity chromatography (Table 1). Protein sequences (N, GP5-M, M 5', M 3', GP5 5', GP5 3', nsp2p) were derived from PRRSV isolate VR-2332 and extended with amino terminal myc tags and carboxyl terminal 6x-his tags, as previously described (Baker et al., 2007). Protein expression and purification were performed as described elsewhere (Baker et al., 2007; Johnson et al., 2007). Briefly, recombinant proteins were over-expressed in *E. coli* strain Rosetta2[DE3] (Novagen, Inc., Madison WI USA) and purified from inclusion bodies by chromatography on Ni-NTA-agarose (Qiagen, Inc.) in buffers containing 6M guanidine hydrochloride. Ni-NTA-purified nucleocapsid and nsp2p were refolded, as described by Buchner, et al. (1992) and Clark (1998). Proteins were concentrated and buffer was exchanged on a cross-flow hollow fiber cartridge (Amersham Biosciences, Piscataway, NJ, USA) or on a YM-3 Amicon® Centriprep centrifugal filter device (Millipore Corporation, Billerica, MA, USA), followed by dialysis (Spectra/Por® MWCO 8000; Spectrum® Laboratories, Greensboro, NC, USA) against 20 mM sodium

phosphate (pH 7.5). The concentrate was clarified by centrifugation and filter-sterilized by passage through a 0.2 μm filter. The concentration of total protein was determined by Bradford assay using BSA as the standard. Proteins were stored at -80°C at approximately 0.5-1 mg/ml in 20 mM Tris HCl, pH 8.0, and 150 mM NaCl.

Protein-specific antibody ELISA's were performed using flat-bottomed, Immulon® 2HB 96-well plates (Thermo Labsystems, Franklin, MA, USA). Wells were coated with 500 ng of recombinant protein (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) in alternative columns. Proteins were appropriately diluted in 15mM sodium carbonate and 35mM sodium bicarbonate and buffered to pH 8.8**.** The optimum concentration of recombinant fusion protein was determined by a checkerboard titration to estimate the antibody concentration generating an optical density of approximately 2.0.

All proteins concentrations and positive control antibody concentrations were identical throughout each run. The positive control was based on convalescent serum collected from one boar infected with a North American field isolate of PRRSV. This positive control serum was used on all ELISA plates and used to mathematically standardize (S/P) ratios among the different proteins to account for differences in the optical density (OD) of the antibody response. Thus, protein ELISAs were standardized to each other so as to provide directly comparable data.

To perform the assay, the antigen-coated plates were incubated for one hr at 37°C and then maintained at 4°C overnight. On the subsequent day, the coated plates were washed 6 times with PBS with 0.05% Tween-20 (PBS-T, vol/vol), blocked by adding 200ul of PBS containing 2% (wt/vol) bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), and allowed to incubate one hr at 37°C. Serum samples were diluted 1:100 in SMD, i.e., PBS containing 0.05% Tween-20 and 5% goat serum (Sigma-Aldrich), and then incubated in the

Table 1. Porcine reproductive and respiratory syndrome virus (PRRSV) proteins and polypeptides used in protein ELISAs[1]

Protein or polypeptide		Description	Nucleotide sequence range[2]
Nucleocapsid	N	Complete 123 amino acid sequence of N	14889-15257
GP5-Matrix protein ectodomain	GP5-M	87 amino acid synthetic peptide with predicted ectodomain regions linked with a neutral petapeptide (GP5 and M)	13875-13979 14085-14114 14375-14431 14561-14584
Matrix protein external	M 5'	32 amino acid synthetic peptide external to the envelope M	14375-14431 14561-14584
Matrix protein internal	M 3'	Carboxy terminal 88 amino acid fragment of M	14633-14896
Envelope glycoprotein 5	GP5 5'	Amino terminal 35 amino acid fragment of mature GP5	13875-13979
Envelope glycoprotein 5	GP5 3'	Carboxy terminal 72 amino acid fragment of mature GP5	14172-14387
Nonstructural protein 2	Nsp2p	Amino terminal 719 amino acid fragment of Nsp2	1339-3495

[1] Baker et al., 2007. Table reprinted with permission of the Journal of Swine Health and Production, ©2008

[2] Nucleotide sequence ranges from Genbank® accession #U87392

antigen-coated wells at 37°C for one hr. After washing 6 times with PBS-T, 100µl of goat anti-swine-IgG horseradish peroxidase-conjugate (KPL, Gaithersburg, MD) was added at a final dilution of 1:500 in SMD and allowed to incubate one hr at 37°C. After another wash, 100ul of the substrate ABTS (KPL, Gaithersburg, MD) was added, then stopped after 15 min by adding 100 µl of ABTS stop solution to all wells (KPL, Gaithersburg, MD). The reactions were read at 405nm absorbance in a multichannel ELISA reader (ELx 808, Bio-Tex Instruments, Inc., Winooski, VT, USA) and the results expressed as a sample-to-positive ratios (S/P).

$$S/P = \frac{(\text{Sample OD in antigen - coated well}) - (\text{Sample OD in uncoated well})}{(\text{Positive control OD in antigen - coated well}) - (\text{Positive control OD in uncoated well})}$$

2.6. ELISPOT

The PRRSV-specific cell-mediated immune response to PRRSV infection was assessed using an enzyme-linked immunosorbent spot (Elispot) assay designed to detect IFNγ-producing PBMCs.

To prepare antigen for the Elispot assay, the original PRRSV inoculum was propagated on MARC-145 cells and semi-purified, as described previously (Yang et al., 1998). In brief, MARC-145 cells were infected with PRRSV and the virus harvested by 3 freeze-thaw cycles (-80°C/37°C) when CPE was evident in 80% of cells. The supernatant was first clarified at 3,500 × *g* for 30 min at 4° C and then overlaid on 20% sucrose and subjected to ultracentrifugation at 120,000 × *g* in a SW28 rotor (Beckman Coulter) for 1.5 hours at 4° C. Thereafter, the pellet was re-suspended in PBS and overlaid onto a discontinuous sucrose gradient (30% and 60%) and centrifuged at 120,000 × *g* in a SW28 rotor for 2.5 hours at 4° C. The band at the interface of the 2 sucrose layers was collected and diluted with cold PBS. The diluted virus was subsequently centrifuged at 90,000 × *g* for 1.5 hours at 4° C, after which the resultant pellet was re-suspended in cold PBS and stored at -80° C until used. The amount of virus in the preparation was determined by microtitration infectivity assay and real-time quantitative reverse-transcriptase PCR (qRT-PCR).

The Elispot assay was conducted in 96-well PVDF-bottom ImmunoSpot® plates (Millipore Corporation). Wells were first coated with 50 µl (6 µg per ml) of primary mouse anti-porcine IFN-γ monoclonal antibody P2G10 (BD Biosciences, San Jose, CA). After overnight incubation at 4° C, the antibody-coated plates were washed 3 times with sterile PBS and inoculated with 5×10^5 cells (50 µl) of PBMCs per well. Thereafter, 50 µl of PRRSV was inoculated on 3 wells of PBMCs from each PRRSV-infected pig and 3 wells of PBMCs from each uninfected pig and incubated for 20 hrs at 37° C. One positive and 3 negative control wells of PBMCs from each pig were included on each plate. Positive control wells were inoculated with 50 µl of 10 µg per ml of phytohemagglutinin (Sigma) and negative control wells were inoculated with 50 µl of MEM (Sigma). After incubation, cells

were removed by washing 6 times with PBS containing 0.05% Tween-80 (PBST, pH 7.2). Then the plates were incubated with 50 µl of biotin-labeled secondary antibody specific for IFN-**γ**, P2C11 (0.5 µg per ml) (BD Biosciences, San Jose, CA) in PBS for one h at 37° C. After 3 washes, the plates were incubated with 50 µl of streptavidin-horseradish peroxidase conjugate (Prozyme, San Leandro, CA, USA) per well for one hr at 37° C. The plates were then washed 3 times with PBST, incubated with 50 µl of substrate (3-amino-9-ethylcarbazole) (Sigma-Aldrich) per well for 30 min at room temperature, washed with distilled water, and dried at 37° C. Thereafter, the reaction was quantified by counting the number of red spots in each well.

In PRRSV-inoculated pigs, the pig-specific Elispot response was adjusted for non-specific reactions by subtracting the number of spots (2 to 5 spots per well) produced in the negative control wells and in the age-matched negative control pigs as follows:

Elispot (adjusted) = [($\bar{x}$ spots in 3 virus-exposed wells from infected pigs - $\bar{x}$ spots in 3 negative control wells from infected pigs) - ($\bar{x}$ spots in virus-exposed wells from uninfected pigs - $\bar{x}$ spots for negative control wells from uninfected pigs)]

2.7. Statistical analyses

Data were analyzed using MedCalc® 9.2.1.0 (MedCalc Software, Mariakerke Belgium), JMP® 6.0 (SAS Institute Inc., Cary North Carolina), and SigmaPlot® 10 (Systat Software, Inc., Richmond, CA). Elispot, qRT-PCR, and FFN values were normalized by logarithmic transformation prior to the analyses.

Initially, descriptive and comparative analyses were conducted to describe the immune responses against PRRSV (DPI 0 to 193). Univariate analyses to examine differences between inoculated and negative control groups and among sampling points were done with one-way ANOVA, with specific contrasts performed using Student's t-test. The associations between the humoral immune responses and Elispot results were analyzed by correlation analysis. Longitudinal effects (time) were evaluated using MANOVA and area under the curve (AUC) analysis. For AUC, the cumulative response over time for each assay

and pig was estimated using the trapezoid rule (Hennen, 2003) and compared by one-way ANOVA. The ability of the various ELISAs to discriminate between PRRSV-inoculated and negative control pigs, i.e., diagnostic performance, was compared using receiver operator characteristic (ROC) curve analyses.

Multivariate logistic regression was used to evaluate the relationship between immune responses and duration of viremia; multiple regression analysis was used to examine the immune response and level of viremia. These analyses were based on a subset of results from PRRSV-inoculated pigs prior to DPI 56. DPI 56 was selected as the point of comparison because 5 of the 10 PRRSV-inoculated animals were no longer viremic on DPI 56.

3. RESULTS

All pigs were determined to be free of PRRSV infection by qRT-PCR analysis of serum samples collected upon arrival into the animal holding facilities at DPI -7 and again on DPI 0. Following exposure to PRRSV, all inoculated pigs were confirmed to be infected on the basis of qRT-PCR-positive results on serum samples collected on DPIs 7 and 14. All negative control animals remained free of PRRSV infection throughout the course of the experiment. A summary of qRT-PCR, Elispot, commercial ELISA (IDEXX Laboratories, Inc.), and FFN results over time are presented in Table 2 and Figure 1. Results from the protein ELISAs (N, GP5 3', GP5 5', M 5', M 3', GP5-M, and nsp2p) are summarized in Figure 2. For each antibody assay, the difference in the means between PRRSV inoculated and negative control animals was significantly different ($p \leq 0.05$) based on one-way ANOVA. Thereafter, the means for each test by DPI were compared using Student's t-test. These results are summarized in Table 3.

Correlations between FFN and antibody ELISA results were tested for the entire period of observation (DPI 0 to 193) and then re-evaluated using a subset of samples from the viremic period of the infection (defined as DPI 0 to 56). For the entire dataset, the strongest correlation to FFN was observed with nsp2p ($r = 0.46$), followed by GP5-M ($r = 0.42$) and the commercial ELISA ($r = 0.33$). Correlation analysis using the subset of results

from the viremic period (≤ DPI 56) showed that the three ELISAs most strongly correlated with FFN were the GP5-M (r = 0.69), nsp2p (r = 0.62), and M 3' (r = 0.58).

Correlation analysis of the commercial ELISA (IDEXX Laboratories, Inc.) and the protein ELISA results was done for the entire period of observation and then separately for the period through DPI 56. For the entire dataset, the strongest correlations with the commercial ELISA were with the N ELISA (r = 0.81), the nsp2p ELISA (r = 0.67), and the M 3' ELISA (r = 0.65). Analysis of the viremic stage of the infection (≤ DPI 56) showed the strongest correlations with the N (r = 0.90), M 3' (r = 0.74) and nsp2p (r = 0.71) ELISAs.

A MANOVA analysis of the response by time found that the longitudinal antibody responses differed significantly among the protein ELISAs ($p < 0.001$). This analysis was appropriate because the magnitude of the response was standardized among the protein ELISAs (see 2.5.3). The commercial ELISA was not included in this analysis because its response was not standardized to the protein ELISAs. Specific contrasts found no difference in the antibody responses measured by the N ELISA and the nsp2p ELISA ($p = 0.24$). Correspondingly, a one-way ANOVA of the cumulative response over time as measured by the AUC found no difference in the responses among the N, nsp2p, and GP5 5' ELISAs.

ROC analyses are typically done to compare the ability of antibody assays to discriminate between infected (positive) and uninfected (negative) groups. In this study, ROC analysis provided a standardized, unit-free method to compare the various ELISAs. As shown in Table 5, the assays that most reliably differentiated PRRSV-inoculated pigs from negative control pigs were the commercial ELISA (AUC = 0.97), the N ELISA (AUC = 0.96), and the M 3' ELISA (AUC = 0.93).

The association between the number of IFNγ-producing PMBCs (Elispot assay) and the humoral immune response or level of viremia (qRT-PCR) was evaluated by Pearson correlation analysis using the complete set of results (DPI 0 to 193). The assays most highly

Table 2. PRRSV viremia (qRT-PCR), fluorescent focus neutralizing assay (FFN), and enzyme-linked immunosorbent spot assay (Elispot) over day post inoculation (DPI)[1]

	FFN[2] (log_2)		qRT-PCR[3] (log_{10})		ELISPOT[4] (log_{10})	
DPI	mean ± SE	Positive pigs[1]	mean ± SE	Positive pigs[1]	mean ± SE	Positive pigs[1]
0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0
7	0.00 ± 0.00	0	7.29 ± 0.40	10	0.00 ± 0.00	0
14	0.06 ± 0.06	1	6.98 ± 0.44	10	2.07 ± 0.19	9
28	0.00 ± 0.00	0	3.56 ± 0.60	9	2.07 ± 0.19	9
42	2.50 ± 0.48	8	1.77 ± 0.53	6	1.97 ± 0.11	9
56	2.30 ± 0.15	10	1.60 ± 0.60	5	1.96 ± 0.10	9
70	3.80 ± 0.39	10	0.59 ± 0.41	2	1.95 ± 0.07	9
84	2.20 ± 0.39	9	0.30 ± 0.30	1	1.98 ± 0.61	9
98	1.60 ± 0.37	7	0.22 ± 0.21	1	1.76 ± 0.22	9
112	1.10 ± 0.39	5	0.00 ± 0.00	0	1.82 ± 0.10	9
126	2.20 ± 0.13	10	0.00 ± 0.00	0	1.69 ± 0.11	9
140	1.20 ± 0.33	6	0.00 ± 0.00	0	ND	ND
154	2.40 ± 0.16	10	0.00 ± 0.00	0	1.77 ± 0.42	9
168	1.80 ± 0.33	8	0.00 ± 0.00	0	ND	ND
182	1.70 ± 0.30	8	0.00 ± 0.00	0	1.82 ± 0.09	9
193	1.80 ± 0.42	7	0.00 ± 0.00	0	ND	ND

[1] Estimates based on 10 PRRSV-inoculated pigs at each sampling

[2] Serum-virus neutralizing antibody quantified using a modified fluorescent focus neutralizing assay (FFN). Positive results based on a cut-off titer of ≥1:4.

[3] PRRSV quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Positive results based a critical threshold (Ct) of ≤39.

[4] Enzyme-linked immunosorbent spot assay (Elispot)

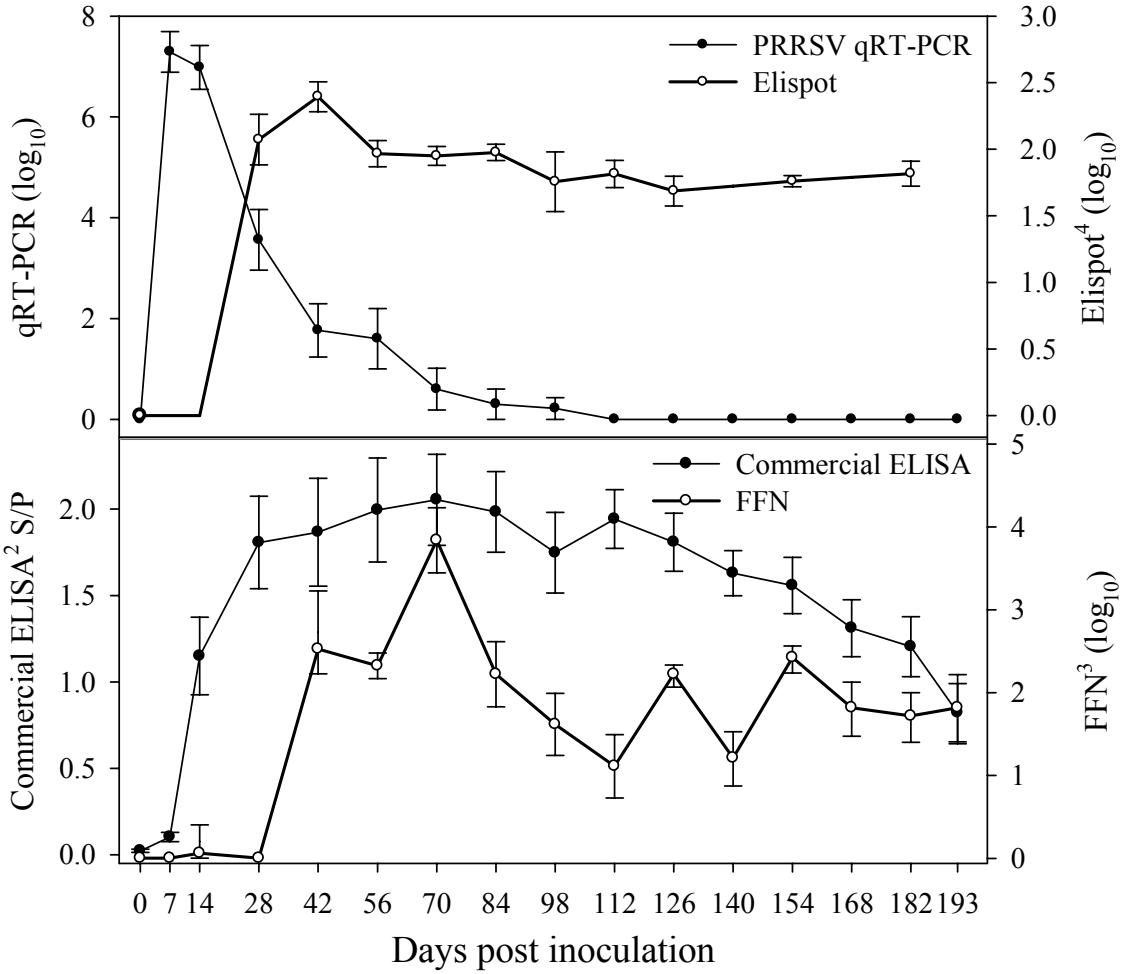

Figure 1. Virological and immunological responses following inoculation of 10 pigs with PRRSV isolate VR-2332. ([1]Means based on 10 PRRSV-inoculated pigs at each sampling; [2]HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA; [3] Serum-virus neutralizing antibody quantified using a modified fluorescent focus neutralizing assay (FFN); [4] Enzyme-linked immunosorbent spot (ELISPOT) assay for detection of IFNγ-producing PBMCs)

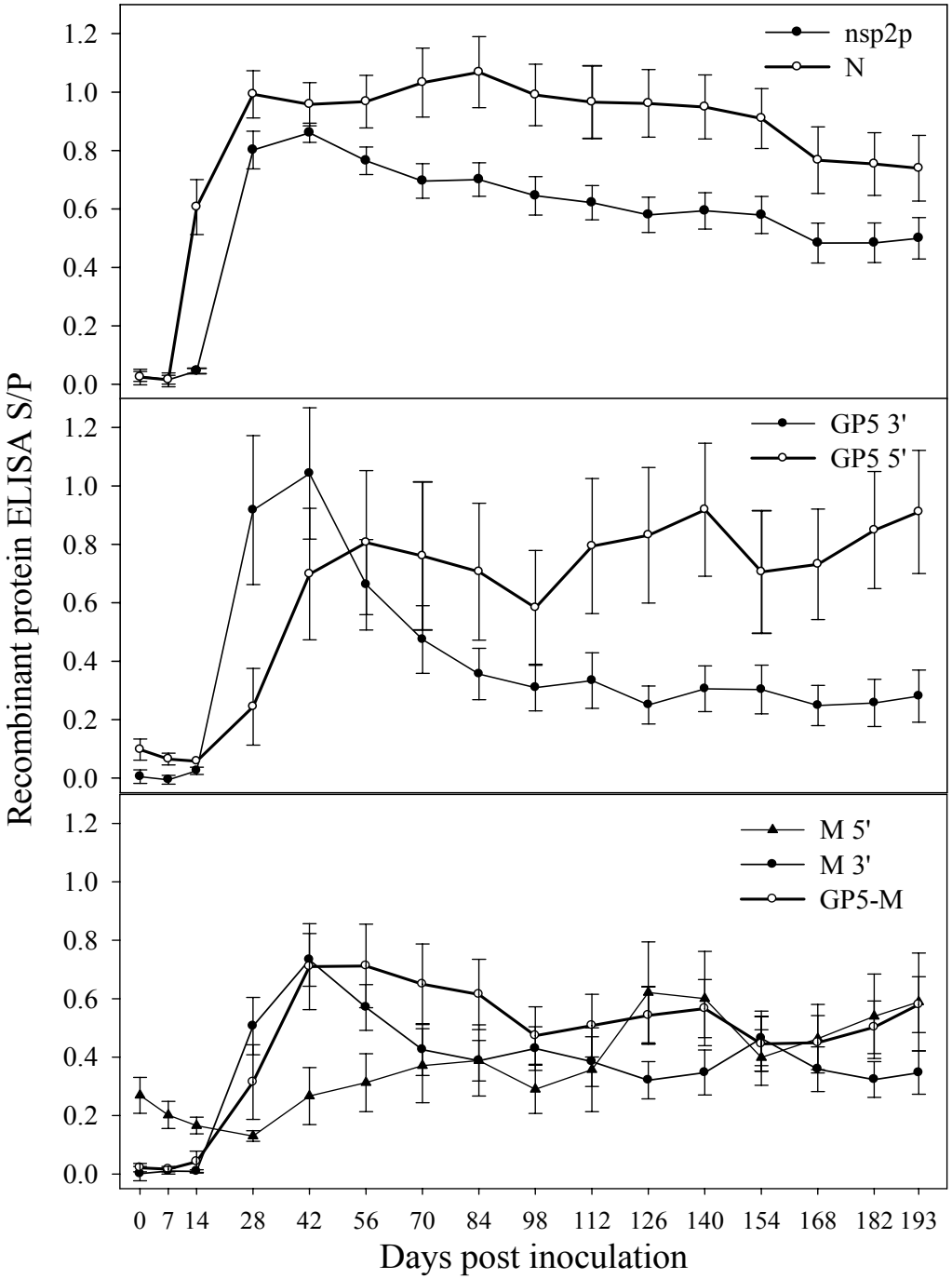

Figure 2. Antibody responses following against specific viral proteins following inoculation of 10 pigs with PRRSV isolate VR-2332. ([1]Means based on 10 PRRSV-inoculated pigs at each sampling)

Table 3. Analysis of anti-PRRSV immune responses by day post inoculation (DPI)

Assay	Significant differences by DPI ($p < 0.05$)[1]
Elispot (IFNγ)[2]	Means at DPIs 0 and 7 differed from means at DPIs 14 to 193.
	Peak response at DPI 42 was significantly greater than all other sampling points.
FFN[3]	Means at DPIs 0 to 28 differed from means at DPIs 42 to 193.
	Peak response at DPI 70 was significantly greater than means at DPIs 56 to 193.
Commercial ELISA[4]	Means at DPIs 0 and 7 differed from means at DPIs 14 to 193. No significant difference between the peak response at DPI 70 and the means at later sampling points.
N ELISA	Means at DPIs 0 and 7 differed from means at DPIs 14 to 193.
	No significant difference between the peak response at DPI 84 and the means at later sampling points.
GP5-M ELISA	Means at DPIs 0, 7 and 14 differed from means at DPIs 28 to 193.
	No significant difference between the peak response at DPI 56 and the means at later sampling points.
M 5' ELISA	No significant difference between the means at any DPI.
M 3' ELISA	Means at DPIs 0 to 14 differed from means at DPIs 28 to 193.
GP5 5' ELISA	No significant difference between the means at any DPI.
GP5 3' ELISA	Responses at DPI 28 and 42 significantly greater than all other DPIs.
Nsp2p ELISA	Means at DPIs 0 to 14 differed from means at DPIs 28 to 193.
	No significant difference between the peak response at DPI 42 and the means at later sampling points.

[1] Specific contrasts performed using Student's t-test
[2] Enzyme-linked immunosorbent spot assay (ELISPOT)
[3] Serum-virus neutralizing antibody quantified using a modified fluorescent focus neutralizing assay (FFN)
[4] HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA

Table 4. Diagnostic performance of antibody ELISAs based on receiver operator characteristic (ROC) curve analysis

ELISA	Rank[1]	AUC[2]	SE[3]	95% CI[4]	Se[5]	Sp[6]	Cut-off[7]
Commercial[8]	A	0.97	0.012	0.93, 0.99	88.1	100.0	0.13
N	AB	0.96	0.013	0.92, 0.98	89.4	100.0	0.08
M 3’	BC	0.93	0.020	0.88, 0.96	83.1	98.7	0.04
GP5-M	CD	0.88	0.027	0.82, 0.92	77.5	96.9	0.25
Nsp2p	D	0.85	0.029	0.80, 0.90	78.1	93.7	0.25
GP5 5'	E	0.79	0.038	0.72, 0.84	75.6	78.1	0.07
GP5 3'	EF	0.73	0.043	0.66, 0.79	63.7	96.9	0.06
M 5’	F	0.62	0.052	0.54, 0.69	52.8	81.2	0.20

[1] Assays with the same letter are equivalent ($p > 0.05$)
[2] Area under the curve (AUC) derived from ROC analysis of cumulative results (0 to 193 DPI)
[3] Standard error for the AUC
[4] 95% confidence interval for the AUC
[5] Diagnostic sensitivity
[6] Diagnostic specificity
[7] Optimized cut-off is the point at which diagnostic sensitivity and diagnostic specificity were maximized as determined by the ROC analysis
[8] HerdChek® PRRS Antibody 2XR Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA

correlated with Elispot were the N ELISA (r = 0.61), the nsp2p ELISA (r = 0.60), and the commercial ELISA (r = 0.56). The correlation between the Elispot response and the remaining assays was weak (r < 0.50). Likewise, correlation analysis found a weak association between Elispot results and level of viremia (qRT-PCR) (r = -0.23). The correlation analyses were repeated using a subset of samples from the viremic stage of the infection, i.e., sampling points DPI 0 to 56. Using these data, the correlation coefficients between ELISPOT results and most antibody assays improved, e.g. N ELISA (r = 0.81), the M 3’ ELISA (r = 0.72), the nsp2p ELISA (r = 0.68), and the commercial ELISA (r = 0.56).

In contrast, the correlation between the number of IFNγ-producing PMBCs (Elispot assay) and virus load (qRT-PCR) weakened (r = -0.08).

Multivariate analyses were performed to evaluate the relationship between the immune response and the level or duration of viremia (qRT-PCR) using results from PRRSV-inoculated pigs prior to DPI 56. This period of time was selected for the analysis because 5 of the 10 PRRSV-inoculated animals were no longer viremic by this time, i.e., these data provided the best likelihood of detecting a difference in immune responses between pigs that cleared virus and those that did not.

Factors impacting the level of viremia were analyzed in a three-step approach. First, simple regression analysis of the protein ELISAs using the level of viremia (qRT-PCR) as the dependent variable found a statistically significant association ($p < 0.05$) with several ELISAs , i.e., GP5 3', GP5 5', GP5-M, M 3', and nsp2p. These variables were tested in stepwise fashion in a multiple regression model, adding variables in order of decreasing statistical significance. The best fit model showed that viremia was predicted by the nsp2 ELISA response (y = 4.83 – 3.09 nsp2, $p = 0.001$). Second, Elispot and FFN responses were tested for an association with the level of viremia using multiple regression analysis. This analysis showed a statistically significant ($p < 0.05$) association between the level of viremia and FFN, but not with Elispot. Third, nsp2p and FFN were evaluated in a final multiple regression model. This analysis determined that the FFN response alone was the best predictor of level of viremia (y = 4.40 – 1.08 FFN, $p < 0.001$).

Factors impacting the duration of viremia were analyzed using the same three-step approach. In the first step, simple regression analysis found no statistical significance between the duration of viremia and the various protein ELISA responses ($p > 0.05$). Thereafter, the protein ELISAs were tested in a step-wise multiple logistic regression model, adding variables in order of decreasing statistical significance at each iteration. The best fit model described the duration of viremia as a function of M 3' and GP5 3' responses (y = -0.30 + 5.87 M 3' - 4.09 GP5 3', $p = 0.0001$). In the second step, Elispot and FFN responses were evaluated using multiple logistic regression analysis with the result that FFN showed a statistically significant ($p < 0.05$) association with the duration of viremia. Third, multiple logistic regression analysis using FFN, M 3' ELISA, and GP5 3' ELISA found that only

neutralizing antibody (FFN) predicted the duration of viremia (y = 1.08 – 0.43 FFN, $p <$ 0.001). Thus, the FFN response was the best predictor of both level and duration of viremia.

4. DISCUSSION

The goal of this study was to evaluate immunological responses as diagnostic predictors of PRRSV infection status. The study was based on an extensive set of samples collected from ten PRRSV-inoculated animals and ten age-matched negative controls for 193 days after inoculation. Samples were assayed for a variety of responses, including PRRSV viremia (qRT-PCR), humoral immune responses, and interferon-γ-secreting cells in blood. Overall, the strengths of the experiment included the prolonged period of sampling, i.e., 193 days, and the number of immunological and virological parameters tested. The study produced an unusually large dataset that was amenable to extensive statistical analysis.

In this study, PRRSV viremia was detected at the first serum sampling on DPI 7 (10 of 10 pigs). One-half of the pigs (5 of 10) resolved the viremia by DPI 56, with the final PCR-positive detected on DPI 98. Although the magnitude of viremia is believed to vary by isolate (Johnson et al., 2004), these results are compatible with the duration of viremia previously reported for isolate VR-2332, the North American prototype PRRSV (Horter et al., 2002).

Interferon-γ is presumed to play an important role in the immune response against PRRSV (Meier et al., 2003). In agreement with previous studies (Diaz et al., 2005; Lowe et al., 2006; Meier et al., 2003), PRRSV-inoculated pigs showed a measureable PRRSV-specific IFN-γ response by DPI 28. In this study, the response reached a plateau at DPI 42, then declined and remained relatively stable level throughout the remainder of the observation period. The findings in this study are consistent with a previous report that the response is lower in persistent than in acute infection (Xiao et al., 2004) and are in contrast to a report that the specific IFN-γ response increases with time after infection (Meier et al., 2003). Statistical analysis showed a weak correlation between the IFN-γ-secreting activity and the magnitude or duration of viremia, but did show a correlation between PRRSV-specific IFN-γ-secreting lymphocytes and the N, M 3', and nsp2 ELISAs. Overall, the value

of the Elispot response as an indicator of viral infection and PRRSV immunity is unclear, but appears weak based on these data.

Neutralizing antibodies were detected in one of 10 pigs at DPI 14, but a clear and definitive neutralizing response was absent until DPI 42 with a peak response at DPI 70. These findings are in agreement with previous publications (Batista et al., 2004; Murtaugh et al., 2002). The role of neutralizing antibodies in the resolution of viremia and development of protective immunity against PRRSV has been a point of discussion (Lopez and Osorio, 2004). Most studies have shown that the production of neutralizing antibodies after experimental inoculation or vaccination is slow and highly variable among pigs (Loemba et al., 1996; Meier et al., 2003). Osorio et al. (2002) showed that exogenous neutralizing antibodies at high titers could protect against clinical reproductive losses in pregnant animals, but would not prevent infection. In this study, multivariate analyses that included all antibody assays and Elispot showed that only neutralizing antibody, as measured by the FFN assay, was able to predict the duration and level of PRRSV viremia.

The protein ELISAs used in this study were based on the three major structural proteins (GP5, M, and N) and one non-structural protein (nsp2) (Table 1). The commercial ELISA (IDEXX Laboratories, Inc.) used to monitor antibody response in this study is the assay most frequently used for detection of PRRSV antibodies, but the precise composition of the antigen is proprietary information. Antibody responses measured by the commercial ELISA (IDEXX Laboratories, Inc.) and the N ELISA showed a good correlation with a history of exposure to PRRSV, i.e., diagnostic sensitivity and specificity, including the long period after the cessation of viremia. These two assays had the additional virtue of early response, i.e., a strong positive response at 14 DPI, whereas most other protein-specific antibody ELISAs detected responses at 28 or 42 DPI. In the case of the GP5 3' and nsp2p ELISAs, once detected, the levels of antibody remained high until the termination of the study. The GP5-M ELISA, a chimeric polypeptide containing all of the putative ectodomains of the PRRSV GP5-M heterodimer that may be critical to viral infection and antibody neutralization, was the ELISA most strongly correlated with the FFN response, followed by the nsp2 and M ELISAs. These results suggest the possibility of developing an ELISA that accurately reflects serum-virus neutralizing antibody levels.

An unanticipated outcome of the study was the variation in antibody responses to specific viral proteins and polypeptides. For example, the endodomain fragment of GP5, the GP5 3' fragment, showed a peak in antibody response at 28 to 42 days after infection, followed by a decline to steady-state levels around 70 to 80 days after infection. The ectodomain portions of the same protein showed a delayed antibody response (Figure 2), reaching a sustained plateau at about 42 days after inoculation and maintaining this level until the end of the study. Thus, the time frame of the GP5 5' response was similar to the FFN response. A related protein, M, which forms a heterodimeric structural complex with GP5, showed a different pattern of immunoreactivity between endodomain (M 3') and ectodomain (M 5') fragments (Figure 2). Although exhibiting a degree of variation, the overall pattern of antibody response against GP5-M was similar to the two functional domains of this protein. The structural proteins N and GP5 3' and the nonstructural protein nsp2p elicited sustained antibody responses suggestive of prolonged antigenic stimulation in lymphoid tissues. The ectodomain fragments of GP5 and the M polypeptide fragments showed a lower level of antibody responses. Thus, their significance as prognostic indicators is reduced.

The overall conclusion from this study is that humoral antibody responses, particularly the commercial ELISA, N ELISA, and M 3' ELISA are the best predictors of prior exposure to PRRSV, but provide little prognostic information regarding the ontogeny of the protective immune response. Cell-mediated immunity, as assessed by IFN-γ-secreting activity, appears to be a poor prognosticator of PRRSV infection status. With respect to differentiation of acute versus persistent infection, a reliable serological indicator remains to be identified.

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Chapter 5. Diagnostic performance of assays for the detection of anti-porcine reproductive and respiratory syndrome virus antibodies in serum and muscle transudate ("meat juice") based on samples collected under experimental conditions

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ABSTRACT

Three assays were evaluated for their ability to detect antibodies against *Porcine reproductive and respiratory syndrome virus* (PRRSV) in porcine muscle transudate ("meat juice") samples. Samples were derived from 91 pigs inoculated with PRRSV isolate VR-2332 and 46 age-matched controls. Serum and muscle (*Musculus longissimus dorsi*) samples were collected from randomly selected animals euthanized at ~14-day intervals from 28 to 202 days post inoculation (DPI). Serum samples were assayed at a dilution of 1:40, and muscle transudate samples were assayed at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40) using a commercial PRRSV antibody enzyme-linked immunosorbent assay (ELISA). Additionally, muscle transudate samples were tested using an indirect fluorescent antibody test (IFAT) at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40). Attempts to assay muscle transudate samples for neutralizing antibodies using a modified fluorescent focus neutralization assay were unsuccessful. Receiver operator characteristic (ROC) curve analyses were used to estimate cut-off thresholds and the associated diagnostic sensitivities and specificities for ELISA and IFAT at each dilution. For ELISA, muscle transudate samples at the ROC-optimized cut-offs were >95% sensitive and 100% specific at each dilution. At a cut-off dilution of ≥1:5, the IFAT diagnostic sensitivity and specificity of muscle transudate was estimated at 63.3% and

100%, respectively. These findings validated the use of muscle transudate samples in PRRSV surveillance programs based on ELISA antibody testing.

INTRODUCTION

Unrecognized prior to the 1980s (Keffaber KK: 1989, Reproductive failure of unknown etiology. Am Assoc Swine Pract Newsletter 1:1-10), *porcine reproductive and respiratory syndrome virus* (PRRSV; order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*) had become endemic by the mid-1990s in most areas of the world where swine are raised, and is currently the most costly infectious disease confronting U.S. swine producers.[18,23] Despite advances in basic and applied research, the control of clinical losses on farms and prevention of the spread of PRRSV between farms has been problematic.[11] Faced with ongoing PRRS losses, the general consensus in North America is that the elimination of PRRSV is the best long-term solution.[3]

Successful disease elimination programs are based on accurate, cost-effective, and timely detection of infected animals and herds. Historically, such efforts have relied on "down-the-road" or on-farm serum testing of individual animals. More recently, muscle transudate ("meat juice") samples (i.e., fluid recovered from frozen muscle as it thaws) have proven to be a useful alternative to serum in epidemiological studies and surveillance for infectious agents.[2,14,16,19-21] Muscle samples are easily collected at slaughter, less costly to collect than serum, and completely avoid the biosecurity risks that on-farm visits present. The performance parameters of diagnostic assays (i.e., diagnostic sensitivity and specificity) are critical to the accurate interpretation of test results. Diagnostic performance has not been reported for PRRSV antibody assays testing muscle transudates. Therefore, the objective of this study was to estimate the diagnostic sensitivity and specificity of a commercial PRRSV enzyme-linked immunosorbent assay (ELISA), an indirect fluorescent antibody test (IFAT), and a modified fluorescent focus neutralization (FFN) assay. Tests were evaluated using muscle transudate samples collected from animals of known PRRSV status in a longitudinal experimental study.

MATERIALS AND METHODS

Experimental design, animal care, and animal handling procedures were approved by the Biosafety (#04-I-028-A) and Animal Care and Use (#9-04-5751-S and #7-05-5933-S) Committees at Iowa State University. The project was conducted according to *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*.[9] Fourteen-day-old (n = 165) PRRSV-negative, Large, white/Landrace cross piglets (50% barrows and 50% gilts) were received at the Iowa State University Livestock Infectious Disease Isolation Facility 1 week prior to the beginning of the experiment. Pigs were derived from 17 litters farrowed on day 162 of a 1000-day production calendar. Only healthy, good-quality piglets were selected for inclusion in the experiment. Individual age-matched pigs were randomly assigned to treatments by blindly selecting ear tags (pigs were ear-tagged upon arrival). Tag numbers 1 - 109 (n = 109) were assigned to the PRRSV-inoculated group; numbers 110 - 165 (n = 56) were assigned to the negative control group. Pigs were divided equally by gender between the infected and control groups. Even-numbered tags were assigned to males and odd-numbered tags were assigned to females.

On day 0, blood samples were collected, and pigs were intramuscularly (IM) inoculated in the neck just behind and below the ear with 1 ml of PRRSV ($1 \times 10^{4.0}$ $TCID_{50}$) diluted in minimum essential medium (MEM).[a] Negative-control pigs were sham inoculated with MEM by the intramuscular route. Following inoculation, animals were euthanized at approximately 14-day intervals for up to 202 days post inoculation (DPI). Five pigs (3 PRRSV-inoculated; 2 negative control) were euthanized at each sampling point from DPI 28 through DPI 112; thereafter through DPI 202, approximately 15 pigs (10 PRRSV-inoculated; 5 negative control) were euthanized per sampling.. Serum and muscle samples (*Musculus* longissimus *dorsi*) were collected from each pig at the time of euthanasia. Among the 165 animals, 28 pigs were excluded from the current study because they were too small to provide adequate-sized muscle samples (i.e., prior to DPI 28), died unexpectedly, or were among a group that was rechallenged with PRRSV on DPI 193. In total, paired serum and muscle transudate samples were available from 137 pigs. This subset consisted of 91 PRRSV-infected pigs and 46 negative-control pigs.

Porcine reproductive and respiratory syndrome virus

Virus strain

The North American prototype PRRSV, ATCC VR-2332,[b] was used in the current study. The complete genomic sequence for VR-2332 has been published.[17] The specific virus isolate used was derived from a plaque-cloned virus recovered from the serum of a pig inoculated with VR-2332.[5]

Virus propagation

To avoid the effects of cell passage, virus was inoculated into a 21-day-old pig. On DPI 7, the pig was euthanized and the serum harvested. For virus propagation, serum was inoculated onto 24-hr-old confluent MARC-145 cells, a clone of the MA-104 cell line considered to be highly permissive to North American (type 2) PRRS viruses.[12] MARC-145 cells were prepared in 162-cm^2 flasks[c] containing MEM growth medium: MEM[a] supplemented with 10% fetal bovine serum (FBS),[a] 50 µg/ml gentamicin,[a] 100 IU/ml penicillin,[a] 100 mg/ml streptomycin,[a] and 0.25 µg/ml amphotericin B.[a] After 24 hr at 37°C in a humidified 5% CO_2 incubator, the MEM growth medium was discarded and the flasks inoculated with 5 ml of serum. After 2 hr at 37°C in a humidified 5% CO_2 incubator, the inoculum was discarded and 50 ml of maintenance medium was added: 50 ml of MEM supplemented with 5% FBS, 50 µg/ml gentamicin, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Thereafter, cells were examined daily for cytopathic effect (CPE). When 75% CPE was observed, the medium was freeze-thawed (-80°C/25°C) and cell lysates were harvested and stored at -80°C.

Virus titration

Virus titrations were done on confluent monolayers of MARC-145 cells in 96-well plates.[c] Cell monolayers were prepared by adding 200 µl of a solution containing 4×10^5 cells per ml suspended in MEM growth medium to each well, after which plates were placed for 24 hr in a 37°C, humidified, 5% CO_2 incubator. Each sample was serially 10-fold diluted in MEM and then 4 wells were inoculated with 100 µl of stock virus at each dilution. Thereafter, plates were incubated at 37°C in a humidified 5% CO_2 incubator for 2 hr, the

inoculum was discarded, and 200 µl of maintenance medium was added to each well. Wells were examined for CPE for up to 2 DPI. At the end of 2 days, the cells were fixed with aqueous 80% acetone solution and stained with fluorescein isothiocyanate-conjugated monoclonal antibody (mAb) SDOW17-F.[d]

Euthanasia protocol

Pigs to be euthanized were first IM administered (1.0 ml per 22.5 kg of body weight) a solution composed of 5 mg tiletamine and 5 mg zolazepam[e] reconstituted with 250 mg xylazine[f] and 250 mg ketamine.[e] When recumbent, pigs were intravenously administered (1.0 ml per kg of body weight) a solution containing 0.2 mg butorphanol,[e] 2.0 mg xylazine,[m] and 2.0 mg ketamine.[e] When a surgical plane of anesthesia was reached, pigs were weighed and then exsanguinated.

Samples and sample processing

Serum and muscle tissue were collected from each pig at the time of euthanasia. Blood samples were collected using a single-use blood collection system[g] and then centrifuged at 1000 × *g* for 10 min. Serum was harvested, aliquoted into 2.0 ml cryovials,[h] and stored at -80°C until tested.

At necropsy, 2 sections (left and right) of the *M. longissimus dorsi* were collected from the lumbar area. The following specific measures were taken to eliminate virus cross-contamination of samples at necropsy: 1) negative-control animals were necropsied before inoculated pigs; 2) carcasses were washed with a disinfectant solution[i]; 3) necropsy instruments were washed, immersed in methanol, and flamed between each tissue collection; 4) specimens were immediately placed in sterile plastic bags[j]; 5) specimens were placed on wet ice immediately after collection, frozen (-20°C), and stored frozen until assayed; and 6) latex gloves were changed between each pig.

While still frozen, the muscle sample from the left side was halved and each half processed separately, resulting in 2 samples per pig. One-half was placed in a sterile plastic bag. The remaining half was minced in a sterile meat grinder[k] while still frozen and then placed in a plastic bag. Thereafter, intact and ground muscle samples were held at 4°C for 8

-12 hr, after which the transudate was collected into a 50 ml centrifuge tube.[e] The pH and quantity of fluid was recorded, then the samples were clarified (1000 × *g* for 10 min), aliquoted into 6 snap-cap tubes,[e] and stored at -80°C until assayed. For testing, serum and muscle transudate sample tubes were randomized, renumbered, and submitted as 1 set of samples.

Antibody assays

Enzyme-linked immunosorbent assay

Serum and muscle transudate samples were tested for the presence of PRRSV-specific antibodies using the HerdChek® PRRS 2XR ELISA[l] in a laboratory meeting the requirements of ISO 17025 and designated an A2LA accredited provider of PRRSV ELISA testing.[m] Four serum standards (high positive, medium positive, low positive, and negative) were run on each test plate. Serum standard test results (i.e., ELISA sample-to-positive (S/P) ratios) were analyzed through statistical process control charting to ensure acceptable variation prior to approval of test results. The same PRRS 2XR ELISA test kit lot was used for all samples tested. Serum samples were assayed at a dilution of 1:40, as recommended by the manufacturer. Muscle transudate samples were assayed at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40) using buffer solution provided with the ELISA kit as the diluent. ELISA results were expressed as S/P values.

Indirect fluorescent antibody test

MARC-145 cells at concentration of 4×10^5 cells per ml (150 µl per well) suspended in MEM[g] supplemented with 10% heat inactivated FBS,[n] were grown in 96-well plates[h] until confluent (2 days). After removing the medium, a PRRSV suspension of the homologous virus (ATCC VR-2332) at a concentration appropriate to produce ~100 foci of infected cells per each well (10^3 - 10^4 $TCID_{50}$ per ml) was added to alternating rows of cells. Thereafter, plates were incubated for 18 - 24 hr at 37°C in a humidified 5% CO2 incubator. The medium was removed and cells were fixed in acetone (80%) for 15 min at 4°C and allowed to dry at room temperature prior to use. For the current study, each sample was assayed by a semiquantitative IFAT at 5 dilutions (1:2, 1: 5, 1:10, 1:20, 1:40). Following dilution in

PBS,[g] 100 µl of each sample dilution were transferred to 2 wells of previously infected cell plates. Plates were then incubated for 30 min al 37°C, washed twice in PBS,[g] and stained using fluorescein isothiocyanate (FITC)-conjugated goat anti-swine IgG.[o] The IFAT response was recorded as the highest dilution providing specific cytoplasmic fluorescence. Positive and negative controls were included in each plate.

Fluorescent focus neutralization assay

Porcine reproductive and respiratory syndrome virus-neutralizing activity in serum and muscle transudate samples was measured using a modified FFN assay. Two-fold dilutions (1:4 - 1:512) of heat-inactivated pig serum and muscle transudate samples were prepared on 96-well plates in MEM[p] and supplemented with 2% horse serum.[n] An equal volume of the homologous virus (ATCC VR-2332) at a concentration of 2×10^3 $TCID_{50}$ per ml was added to each sample and incubated for 1 hr at 37°C and then transferred to a 96-well plate containing confluent MARC-145 cells. After 24 hr, the plates were fixed in 80% acetone,[e] stained with FITC-conjugated mAb SDOW17 FITC,[q] with 5% horse serum diluted 1:100 in PBS, and read under an inverted fluorescent microscope. Neutralizing titer was reported as the last dilution that showed 90% or greater reduction in the number of fluorescent foci.

Statistical analysis

Data was analyzed using MedCalc® 9.2.1.0[r] and SigmaPlot® 10.[s] Results were expressed in descriptive parameters and analyzed by correlation analysis and ANOVA, with specific contrasts performed using 2-tailed Student's t-test.

Receiver operator characteristic (ROC) curve analyses were used to estimate diagnostic sensitivities and diagnostic specificities by cut-off using the cumulative data and without considering the effect of time post inoculation. The ROC analyses were conducted on the basis of known PRRSV infection status of the animals. Test accuracy was assessed on the basis of the estimated area under the curve (AUC) generated by plotting test sensitivity (Y-axis) against 1-specificity (X-axis) for all possible cut-off thresholds. Essentially, the ROC analysis evaluated all possible values of diagnostic sensitivity and specificity and

identified the optimized cut-off, i.e., the point at which both sensitivity and specificity were maximized. In addition, specific cut-offs and their effect on sensitivity and specificity were considered in the analysis.

To evaluate the effect of time on diagnostic sensitivity, the estimated diagnostic sensitivity for each DPI was modeled by moving average (3 data points) in a 5-parameter logistic model with a derivation of the equation expressed as:

$$y = d + a\text{-}d/[1+(x/c)^b]^g$$

where (a) is the estimated response at zero DPI, (b) is the slope, (c) is the mid-range DPI, (d) is the estimated response at infinite DPI and (g) is asymmetry.

Thereafter, diagnostic sensitivity and specificity estimates from the 5-parameter logistic model were used to estimate the probability of correctly classifying a herd as PRRSV-negative or -positive over a range of population prevalences (0% to 40%) and for a range of ELISA S/P cut-offs (0.1, 0.2, 0.3, 0.4) using a herd sample size of either 5 or 10 muscle transudate samples. Calculations were performed using HERDACC version 3 software[t] and were based on a theoretical population of 1000 animals. Positive herds were assumed to have become infected with PRRSV approximately 98 days earlier.

RESULTS

Blood was collected from all pigs upon arrival to the Iowa State University animal holding facilities and prior to PRRSV inoculation on DPI 0. *Porcine reproductive and respiratory syndrome virus*-negative status was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) on these samples. All PRRSV-inoculated pigs were confirmed to be productively infected on the basis of positive PRRSV qRT-PCR results on DPI 7 and 14. All negative control animals were shown to have remained free of PRRSV-infection throughout the observation period on the basis of ELISA testing.

Serum antibody assays

Serum enzyme-linked immunosorbent assay

Using the manufacturer's recommended cut-off (S/P ≥ 0.4), all pigs were seronegative for PRRSV antibody at DPI 0 (mean S/P = 0.04; range: 0.00 - 0.23) and all negative-control pigs tested negative throughout the study (mean S/P = 0.02; range: 0.00 - 0.09). ELISA results for serum samples collected at the time of euthanasia from 91 PRRSV-inoculated animals (DPI 28 - 202) are reported in Table 1. From DPI 28 - 98, 100% of pigs were ELISA positive, with S/P means ranging from 1.27 to 1.88. From DPI 112 - 189, 87.5% of pigs were positive, with S/P means by DPI ranging from 0.80 to 1.89. At DPI 202, 9 of 9 pigs tested ELISA positive (mean S/P = 0.91). Cumulatively, serum samples from 91.2% (83/91) of PRRSV-inoculated pigs were ELISA positive at euthanasia. The results of the ROC analysis on the cumulative ELISA data are reported in Table 2. Using the manufacturer's recommended cut-off (S/P ≥ 0.4), diagnostic sensitivity and specificity were estimated at 91.2% (95% confidence interval [CI]: 83.4, 96.1) and 100.0% (95% CI: 92.3, 100.0), respectively. Using the infection status of the animals as the basis for the analysis, the ROC analysis determined that the optimized cut-off was S/P ≥ 0.3. At the ROC-optimized cut-off, diagnostic sensitivity and specificity were estimated at 93.3% (95% CI: 86.2, 97.5) and 100.0% (95% CI: 92.3, 100.0), respectively.

Serum fluorescent focus neutralization assay

Serum FFN results are reported in Table 1. Based on a cut-off of ≥1:4, all pigs were seronegative for PRRSV at day 0 and all negative-control pigs remained negative throughout the study. Neutralizing antibodies were first detected on DPI 42, with the peak response at DPI 56 (mean FFN antibody titer: 1:9.3). At DPI 202, 8 of 9 pigs tested FFN positive. Overall, PRRSV-specific neutralizing antibodies were detected by FFN in 59.3% (54/91) of

Table 1. Analysis of serum: PRRSV ELISA and fluorescent focus neutralization (FFN) antibody response by day post inoculation (DPI).

DPI	Pigs (n)		ELISA S/P[a] value		FFN antibody titer[b]	
	Sampled	ELISA positive	Mean	Standard deviation	Mean	Standard deviation
28	3	3	1.87	0.70	0.0	0.0
42	3	3	1.88	0.21	1.3	2.3
56	3	3	1.27	0.71	9.3	6.1
70	3	3	1.62	0.82	1.3	2.3
84	3	3	1.43	0.82	2.7	2.3
98	3	3	1.58	0.76	4.0	4.0
112	3	2	0.80	0.48	1.3	2.3
119	11	11	1.89	0.89	6.2	9.0
133	10	10	1.28	0.59	0.0	0.0
147	10	10	1.52	0.89	3.2	1.7
161	10	8	1.26	0.81	4.0	3.3
175	10	7	0.95	0.86	4.0	3.3
189	10	8	0.97	0.68	4.4	3.5
202	9	9	0.91	0.43	8.4	6.5

[a] HerdChek® PRRS 2XR ELISA.[1] Manufacturer's recommended cut-off for positive response is sample-to-positive control (S/P) ratio ≥0.4.

[b] FFN means reported as the reciprocal of the serum dilution. Reciprocal of the FFN cut-off for positive response is ≥4.

Table 2. Analysis of serum and muscle transudate: Receiver operating characteristic (ROC) analysis of cumulative PRRSV ELISA sample-to-positive control (S/P) data

Specimen	AUC[a]	Diagnostic Sensitivity	Diagnostic Specificity	ELISA[b] S/P cut-off
Serum				
1:40 dilution	0.997 (0.99, 1.00)[c]	91.2 (83.2, 96.0)[c]	100.0 (92.3, 100.0)[c]	0.400
1:40 dilution	0.997 (0.99, 1.00)	93.3 (86.0, 97.5)	100.0 (92.3, 100.0)	0.300[d]
Muscle transudate				
1:2 dilution	0.981 (0.96, 1.00)	95.6 (89.0, 98.7)	100.0 (92.3, 100.0)	0.026[d]
1:5 dilution	0.996 (0.99, 1.00)	96.7 (90.6 , 99.3)	100.0 (92.3, 100.0)	0.034[d]
1:10 dilution	0.999 (0.99, 1.00)	97.8 (93.9, 99.7)	100.0 (92.3, 100.0)	0.025[d]
1:20 dilution	0.992 (0.98, 1.00)	98.9 (93.9, 99.9)	100.0 (92.3, 100.0)	0.023[d]
1:40 dilution	0.981 (0.96, 1.00)	96.7 (90.6, 99.3)	100.0 (92.3, 100.0)	0.023[d]

[a] Area under the curve

[b] HerdChek® PRRS 2XR ELISA.[l]

[c] 95% confidence interval

[d] Optimum ELISA S/P cut-off calculated using MedCalc®.[r]

inoculated pigs. Analysis of ELISA S/P values versus $\log_2$ transformed FFN antibody titers estimated the coefficient of correlation (r) at 0.136**.**

Muscle transudate antibody assays

Muscle transudate characteristics

M. longissimus dorsi muscle specimens from individual pigs weighed an average of 249 grams, with a range by DPI of 74 g (DPI 28) to 465 g (DPI 202). Muscle transudate pH ranged from 6.5 to 7.0 in 133 of 137 (97%) samples, with the exception of 4 samples in which the pH was ≤6.0. The volume of muscle transudate recovered from muscle tissue averaged 10% of the weight of the specimen. Approximately 10% of the transudate samples were untestable at a 1:2 dilution because of coagulation.

Muscle transudate enzyme-linked immunosorbent assay results

Transudate from intact and ground muscle specimens were assayed independently by ELISA. The results were compared for each sample dilution (1:2, 1:5, 1:10, 1:20, 1:40) to determine if either protocol produced a larger harvest of ELISA-detectable antibody. Although transudate from ground muscle specimens tended to produce slightly higher ELISA S/P values, no statistically significant difference was detected (paired Student's t-test) in the ELISA S/P values between intact and ground meat specimens using $p = 0.05$ as the criterion. Since the results were equivalent, the means of the ground and intact muscle transudate ELISA results were calculated for each pig and used in subsequent statistical analyses. The association between serum ELISA and muscle transudate ELISA values was evaluated by correlation analysis for each muscle transudate dilution. The correlation coefficients (r) ranged from 0.64 (1:2 dilution) to 0.80 (1:5 dilution). The cumulative ELISA diagnostic performance for muscle transudate was evaluated by ROC analysis for each dilution (Table 2). Diagnostic sensitivities for a range of S/P cut-off values (0.05 - 0.40) is presented for all dilutions (1:2, 1:5, 1:10, 1:20, 1:40) in Table 3. In all cases and for all cut-off values, diagnostic specificity was estimated at 100%.

To evaluate the effect of time post inoculation on ELISA performance, the calculated diagnostic sensitivities of meat transudate dilutions 1:5 and 1:10 were analyzed in a 5-parameter logistic regression model by DPI at 4 S/P cut-offs (Fig. 1). The 1:5 and 1:10 dilutions were selected for analysis because they did not exhibit the sample-handling problems (coagulation) associated with the 1:2 dilution and they provided a stronger S/P response than other dilutions (1:20, 1:40). As shown in Figure 1, the 1:5 dilution provided better diagnostic sensitivity than the 1:10 dilution for any S/P cut-off. At a 1:5 dilution, the model predicted a diagnostic sensitivity of 96.3%, 86.3%, and 77.8% for S/P cut-offs 0.1, 0.2, and 0.3, respectively, for DPI 28 - 112 (Table 4). The cut-off of 0.4 resulted in an estimated diagnostic sensitivity of 73.5% at DPI 28 with subsequent declines in sensitivity at each sampling point, thereafter.

Table 3. Analysis of muscle transudate: PRRSV ELISA[a] diagnostic sensitivity for 5 dilutions by sample-to-positive control (S/P) cut-off

S/P cut-off	Diagnostic Sensitivity (95% CI) by Dilution [b]				
	1:2 dilution	1:5 dilution	1:10 dilution	1:20 dilution	1:40 dilution
0.05	92.3 (84.8, 96.9)	95.6 (89.1, 98.8)	91.2 (83.4, 96.1)	93.4 (86.2, 97.5)	91.2 (83.4, 96.1)
0.06	92.3 (84.8, 96.9)	92.3 (84.9, 96.9)	86.8 (78.1, 93.0)	91.2 (83.4, 96.1)	89.0 (80.7, 94.6)
0.07	91.2 (83.4, 96.1)	92.3 (84.9, 96.9)	83.5 (74.3, 90.5)	90.1 (82.1, 95.4)	85.7 (76.8, 92.2)
0.08	90.1 (82.1, 95.4)	90.1 (82.1, 95.4)	81.3 (71.8, 88.7)	86.8 (78.1, 93.0)	84.6 (75.5, 91.3)
0.09	90.1 (82.1, 95.4)	89.0 (80.7, 94.6)	75.8 (65.7, 84.2)	85.7 (76.8, 92.2)	81.3 (71.8, 88.7)
0.10	87.9 (79.4, 93.8)	89.0 (80.7, 94.6)	73.6 (63.3, 82.3)	82.4 (73.0, 89.6)	79.1 (69.3, 86.9)
0.20	73.6 (63.3, 82.3)	65.9 (55.3, 75.5)	52.7 (42.0, 63.3)	58.2 (47.4, 68.5)	54.9 (44.2, 65.4)
0.30	60.4 (49.6, 70.5)	54.9 (44.2, 65.4)	34.1 (24.5, 44.7)	49.5 (38.8, 60.1)	40.7 (30.5, 51.5)
0.40	49.5 (38.8, 60.1)	44.0 (33.6, 54.8)	23.1 (14.9, 33.1)	31.9 (22.5, 42.5)	29.7 (20.5, 40.2)

[a] HerdChek® PRRS 2XR ELISA[1]

[b] Diagnostic specificity estimated at 100% (95% CI 93.6, 100) for all dilutions and cut-offs evaluated.

Muscle transudate indirect fluorescent antibody test results

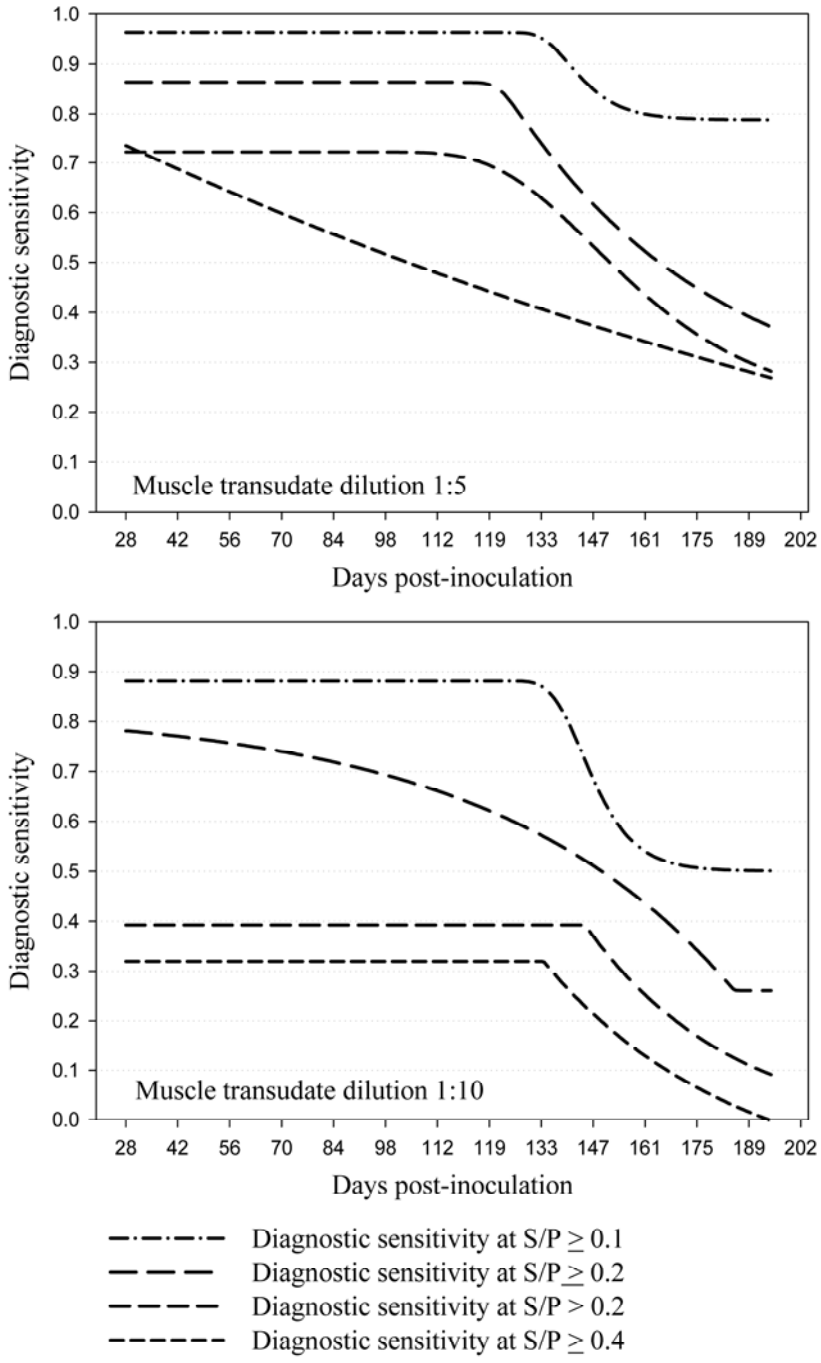
All negative control samples tested <1:5 by IFAT. In the inoculated group, 74.4% of samples showed specific fluorescence at a dilution of 1:2, 63.3% at a dilution of 1:5, and 43.3% at 1:8. Using PRRSV inoculation to define status, an ROC analysis of the cumulative data estimated the optimized cut-off at ≥1:5, with an associated diagnostic sensitivity of 63.3% (95% CI: 53.0, 73.6) and specificity of 100% (95% CI: 92.3, 100.0). To evaluate the effect of time post inoculation on diagnostic sensitivity, the IFAT diagnostic sensitivity was modeled by moving average in a 5-parameter logistic regression model by day post inoculation. As shown in Table 4, a cut-off of ≥1:5 resulted in an estimated diagnostic sensitivity of 100% at DPI 28 with subsequent declines in sensitivity at each sampling point, thereafter.

Table 4. Analysis of muscle transudate at 1:5 dilution: Predicted PRRSV ELISA and indirect fluorescent antibody test (IFAT) diagnostic sensitivity by cut-off by day post inoculation (DPI) as estimated in a 5-parameter logistic regression model

DPI	ELISA[a] S/P cut-off (1:5 dilution)				IFAT
	≥0.1	≥0.2	≥0.3	≥0.4	Cut-off ≥ 1:5
28	96.3%	86.3%	77.8%	73.5%	100.0%
42	96.3%	86.3%	77.8%	68.7%	98.3%
56	96.3%	86.3%	77.8%	64.2%	94.9%
70	96.3%	86.3%	77.8%	59.8%	90.8%
84	96.3%	86.3%	77.8%	55.6%	85.5%
98	96.3%	86.3%	77.8%	51.7%	79.3%
112	96.3%	86.3%	77.6%	47.8%	72.3%
119	96.2%	86.3%	73.2%	46.0%	68.7%
133	95.9%	81.2%	64.5%	42.4%	61.5%
147	91.3%	67.5%	56.3%	39.0%	55.2%
161	81.5%	56.7%	48.3%	35.7%	50.5%
175	79.2%	48.3%	40.8%	32.5%	47.7%
189	78.9%	41.8%	33.5%	29.5%	46.4%
202	78.8%	37.1%	27.1%	26.8%	46.1%

[a] HerdChek® PRRS 2XR ELISA.[1]

Figure 1. Analysis of muscle transudate: predicted PRRSV ELISA diagnostic sensitivity by day post inoculation at 4 sample-to-positive control (S/P) cut-off levels as estimated in a 5-parameter logistic regression model.



Muscle transudate fluorescent focus neutralization results

The first step in the FFN assay is heat inactivation of samples. For muscle transudate samples, this step produced total or partial clotting of the samples. In addition, unacceptably high background levels were observed in seronegative samples. Therefore, it was not possible to produce acceptable results with this assay.

Analysis of herd-level diagnostic sensitivity and specificity

The probability of classifying a population of 1000 animals as PRRSV-negative or -positive using either 5 or 10 muscle transudate samples was calculated using HERDACC version 3 software[t] for a range of ELISA S/P cut-offs and across a range of in-herd prevalences (0% to 40%). Positive results at zero prevalence provide an estimate of the probability of false-positive herd classifications for the parameters described. The analysis was based on the estimates derived from this study, i.e., the diagnostic sensitivity of the ELISA was assumed to be 96.3%, 86.3%, 77.8%, and 51.7% at S/P cut-offs of 0.1, 0.2, 0.3, and 0.4, respectively, and the diagnostic specificity of ELISA was assumed to be 99.5%. Since independent estimates of the diagnostic specificity of the PRRS ELISA on meat juice were not available, this assumption was based on the sensitivity estimates derived from this study and a presumed misclassification error rate of five samples per thousand for true-negative samples. As shown in Table 5, in-herd prevalence, ELISA cut-off, and the number of samples per herds dynamically impact the likelihood of detecting PRRSV-positive herds. Among true PRRSV-negative herds (zero prevalence), 2.5% were mis-identified as infected with a sample size of 5 and 4.9% with 10 samples.

DISCUSSION

Muscle transudate ("meat juice") is a fluid mixture of lymph, serum, and intracellular liquid.[20] Muscle transudate has been shown to be an effective and practical sample for surveillance of salmonella, [20,21] Suid herpesvirus 1 (commonly known as Pseudorabies virus),[14] Yersinia enterocolitic,[21] Trichinella spiralis,[2] and PRRSV.[16] Porcine muscle

Table 5. Probability of detecting one or more PRRSV-positive samples in a swine population as a function of prevalence, number of muscle transudate samples tested, and ELISA cut-off[a]

Prevalence x samples	Probability of ≥ 1 positive sample by ELISA sample-to-positive control (S/P) cut-off			
	≥0.1	≥0.2	≥0.3	≥0.4
0% prevalence (false positive rate)				
5 samples	0.025	0.025	0.025	0.025
10 samples	0.049	0.049	0.049	0.049
5% prevalence				
5 samples	0.239	0.218	0.202	0.146
10 samples	0.421	0.390	0.364	0.271
10% prevalence				
5 samples	0.413	0.380	0.349	0.251
10 samples	0.657	0.617	0.577	0.440
20% prevalence				
5 samples	0.667	0.623	0.583	0.433
10 samples	0.890	0.859	0.827	0.679
30 % prevalence				
5 samples	0.823	0.782	0.742	0.580
10 samples	0.969	0.953	0.934	0.825
40% prevalence				
5 samples	0.915	0.883	0.849	0.693
10 samples	0.993	0.986	0.977	0.906

[a] Estimates calculated using HERDACC version 3[t] for a theoretical population of 1000 animals infected with PRRSV approximately 98 days earlier.

[b] Diagnostic specificity assumed to be 0.995.

specimens and herd identifiers are easily collected at slaughter and transudate is readily recovered by thawing the frozen sample. Diaphragmatic muscle (M. diaphragma crura) is commonly collected for recovery of muscle transudate,[7,20,21] but M. sterno-mastoideus has also been used.[8,16] The possible effect of muscle type on the concentration of total antibody or antibody isotype in transudate has not been evaluated; thus, the selection of muscle is dictated by practical considerations (e.g., the ease with which the specimen may be collected and its value). Musculus longissimus dorsi samples were used in the present study because the large size of the muscle made it possible to recover an adequate volume of muscle transudate from small pigs and because the pigs were precluded from entering the food chain.

In the current study, muscle transudate and serum samples from 91 pigs inoculated with PRRSV isolate VR-2332 and 46 age-matched negative controls were tested in 3 PRRSV antibody assays. Samples were collected from randomly selected animals euthanized at approximately 14-day intervals from 28 to 202 DPI. Serum samples were assayed at a dilution of 1:40 and muscle transudate samples were assayed at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40) on a commercial PRRSV ELISA.[p] Likewise, muscle transudate samples were tested on an IFAT at 5 dilutions (1:2, 1:5, 1:10, 1:20, 1:40). Attempts to assay muscle transudate samples for neutralizing antibodies using a modified FFN assay were unsuccessful.

A ROC analysis of the cumulative serum ELISA data estimated the diagnostic sensitivity and specificity of the test at 91.2% (95% CI: 83.4, 96.1) and 100.0% (95% CI: 92.3, 100.0), respectively. This estimate is somewhat lower than the sensitivity of 97.2% (95% CI: 94.7, 98.7) and specificity of 100% (95% CI: 99.1, 100) previously reported.[10] An explanation for the difference in estimates is that the serum samples used in the present study included a substantial number of specimens from convalescent and chronic stages of the infection (i.e., up to 202 DPI). As reported previously,[1,13,15,22] serum neutralizing antibody response appear ≥4 weeks following inoculation. In agreement with previous studies,[1,15] the PRRSV FFN assay was less sensitive than ELISA. Neutralizing antibodies were first detected on DPI 42, with the peak response at DPI 56 (mean FFN antibody titer: 1:9.3). At DPI 202, 8 of 9 pigs tested FFN positive. Overall, PRRSV-specific neutralizing antibodies were detected by FFN in 59.3% (54/91) of inoculated pigs.

The overarching objective of the current study was to arrive at recommendations for testing muscle transudate for the presence of PRRSV antibodies. An underlying assumption of the discussion is that muscle transudate samples will be used in the surveillance of PRRSV infection, not in routine diagnostics. For surveillance, diagnostic specificity is paramount because false-positive responses are costly in terms of both follow-up costs and erosion of producer confidence in the program.

Initially, ELISA results using ground and intact muscle samples were compared to determine if the concentration of antibody could be increased through processing. Although a comparison of results by DPI showed that transudate from ground muscle samples produced slightly higher ELISA S/P ratios than transudate from intact muscle samples, no statistically significance difference was detected. For that reason, the statistical analyses were run on the means of these results.

A comparison of results by DPI showed that transudate from ground muscle samples produced slightly higher ELISA S/P ratios than transudate from intact muscle samples, but no statistically significance difference was detected. Thus, these results justify the current practice of collecting transudate from intact muscle samples.

Muscle transudate samples tended to coagulate at the lowest dilution tested (1:2); therefore, it is advisable to use assay samples at dilutions >1:2. For all muscle transudate dilutions tested (1:2, 1:5, 1:10, 1:20, 1:40), ELISA diagnostic specificity approached 100%, suggesting that nonspecific reactions are not the major consideration in selecting the optimal testing dilution. A comparison of ELISA diagnostic sensitivity by DPI showed that the 1:5 dilution was more sensitive over a longer period of time post-inoculation than dilutions of 1:10 (Fig. 1), 1:20, or 1:40. Therefore, the 1:5 dilution appears to be the best choice for testing muscle transudates under the conditions described in the current study. For muscle transudate assayed at a dilution of 1:5, the ROC-optimized ELISA S/P cut-off of 0.034 resulted in at diagnostic sensitivity of 96.7% (Table 2). However, this cut-off value is too close to S/P value of zero to accommodate the normal variability in test response expected under routine diagnostic testing conditions. For this reason, diagnosticians may consider alternative ELISA S/P cut-offs of 0.1, 0.2, and 0.3. These cut-offs appear to provide acceptable diagnostic sensitivity when assaying muscle transudate at a 1:5 dilution (Table 4).

In addition, the use of higher cut-offs may be justified by the fact that muscle transudate sampling for surveillance is generally done on a population basis. Therefore, the loss of assay sensitivity is offset by the fact that samples are collected repeatedly over time from the same production sites and multiple samples are taken from each population (Table 5).

The PRRSV IFAT was run on muscle transudate samples because it has been used extensively as a confirmatory test.[13] The IFAT is considered to be highly specific, but diagnostic sensitivity is affected by a variety of factors, including the extent to which the PRRSV isolate used in the assay differs antigenically from the isolate that infected the pig.[6] To control for this source of variability, the homologous virus (ATCC VR-2332) was used in the IFAT. At a cut-off of ≥1:5, the cumulative diagnostic sensitivity of the test was 63.3% and the specificity of the test was 100%. An evaluation on the basis of DPI showed that IFAT is diagnostically sensitive early in the infection, but loses sensitivity over time (Table 4). Therefore, in addition to the other factors that affect IFAT performance, time post infection will be a consideration in the interpretation of IFAT-negative results on meat transudate samples.

Overall, this data further substantiates the use of muscle transudate samples collected at slaughter for surveillance, and provides guidance and performance estimates for using the PRRSV ELISA and IFAT assays described herein. Particularly in the case of the PRRSV ELISA, a stronger S/P response in positive samples would be desirable in order to increase the differentiation between negative and positive muscle transudate samples and, thereby, increase the S/P cut-offs. Future research should focus on this objective. For the ELISA, this might be achieved by increasing the incubation time to increase antibody-antigen binding or achieve the desired effect by diluting the positive control. The former increases the S/P response by increasing the sample response (i.e., ↑S) and the latter by decreasing the positive control response (i.e., ↓P). This approach has been used to adapt other ELISAs to samples containing low levels of antibody (e.g., oral fluids).[4]

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SOURCES AND MANUFACTURERS

a. Sigma-Aldrich, St. Louis, MO.

b. American Type Culture Collection, Manassas, VA.

c. Corning Inc., Corning, NY.

d. Rural Technologies Inc., Brookings, SD

e. Fort Dodge Animal Health, Fort Dodge, IA.

f. LLOYD Laboratories, LLOYD Inc. Shenandoah, IA.

g. Kendall Co., Mansfield, MA.

h. Fisher Scientific Co., Hanover Park, IL.

i. Pharmacal Research Laboratories Inc., Naugatuck, CT.

j. Nasco International Inc., Fort Atkinson, WI.

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l. IDEXX Laboratories Inc., Westbrook, ME.

m. Boehringer Ingelheim Vetmedica, Inc., Health Management Center, Ames, IA.

n. Atlanta Biologicals Inc., Lawrenceville, GA.

o. Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.

p. Invitrogen Corp., Carlsbad, CA.

q. South Dakota State University, Brookings, SD.

r. MedCalc® software, Mariakerke, Belgium.

s. Systat Software Inc., San Jose, CA.

t. HERDACC Version 3 ©1995, David Jordan (http://www.vetschools.co.uk/EpiVetNet/Sampling_software.htm).

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Chapter 6. General Conclusions

As a consequence of the huge economical impact that PRRS has been producing on the swine industry, considerable information has been available in regard of it. Two of the greatest obstacles in the control and prevention of PRRS are the capacity of the virus to become persistent in the production system and the inability of current diagnostic tests to accurately assess the immunological and virological status of naturally infected and vaccinated pigs.

Today, information about the virus replication, immunity, and virus persistence; is available; however, most of the information about virus replication and immune response evaluation has been performed in small numbers of infected pig followed for short period of time. In the other hand, information about persistence came from epidemiological studies in large population at field level. In this work, we never expected to answer all questions related to persistent infection, but our study represent represents a thorough analysis of virus replication and immunity in a relatively large population; from acute infection through persistence and clearance. In addition, it was an opportunity to find new diagnostic assays that can be used to assess PRRSV immunity and persistent infection.

The strengths of this work are: the involving of more than 100 pigs under controlled conditions and the following of them for more than 200 days post inoculation; the inclusion of many conventional virological and immunological tests as a prognosis towards either clearance or persistence of the virus that could be tested and analyzed to provide if single or in combination could provide prognosis of clearance or persistence of the virus; and the extreme care that was taken to avoid the cross contamination among pigs and among the different tissue samples collected from the animals.

In the other hand, the main weakness of this experiment is that the controlled conditions in which it was developed, are quite different than the conditions were the pig are hold at commercial farms. One more weakness is related with the PRRS virus characteristics of highly mutation rates reported in experimental and field studies. We use the American prototype PRRSV isolate and although other published studies, reported similar period of

persistence using different PRRSV isolates, we will never know if our result could be different if other PRRSV isolate were used.

In the first section of this thesis, we tried to provide better estimates of the virological and correlate of PRRSV persistence or clearance. Our results demonstrated that infectious virus is able to persist in populations for a longer period of time than previously thought and that PRRSV persistence can be predicted neither by virus load estimated by qRT-PCR nor immune response estimated by ELISA results; however, the presence of neutralizing antibodies contribute significantly to predict the persistence of the virus. In addition, evaluation of different PRRSV PCR assays on serum, suggested that nested PCR directed to ORF7 is one option of prognosis of the persistence or clearance of the virus. One specific experiment to validate the feasibility of the ORF7 nested PCR as and option to determine presence of infectious virus is required

One interesting outcome from this experiment was the effect of the PRRSV virus replication on the growth of the pigs. During the curse of the experiment was apparent the difference in growth rate and body weight uniformity between infected and control pigs. After discarding the presence of PCV2 and in absence of lesions suggestive of neither mycoplasmal nor bacterial respiratory diseases, we conclude that PRRSV can affect significantly the growth rate of the pigs. In a intent to explain this finding, analysis of possible factors, suggest that duration and magnitude of the viremia is associated with the presence of pigs with low growth rate.

In the experiment included in chapter 3, expanding the current knowledge about the possible role of meat in PRRSV transmission, was demonstrated that the detection of PRRSV on meat samples and other tissues is possible if tests as RT-PCR are used for theses studies; however, our results also demonstrated that detection of PCR positive samples don't mean that neither the pork meat contains infectious virus nor the virus could be transmitted by ingestion. Because the pigs in this experiment were infected at early age, a long period of time to reach the market age, further studies should be focused on determine if infecting pigs at similar ages than occur in farm conditions could repeat or contrast our result.

The experiment in chapter 4 was focused to describe the descriptive study the humoral immune and cell-mediated responses of PRRSV-infected pigs and evaluate to these

responses as diagnostic predictors of PRRSV infection status. One conclusion from this experiment was that the presence of neutralizing antibodies affect the duration and level of viremia in the early stage of PRRSV infection. One more was that those humoral antibody responses, particularly the commercial ELISA, N ELISA, and M 3' ELISA are the best predictors of prior exposure to PRRSV, but provide little prognostic information regarding the ontogeny of the protective immune response.

Finally, because the programs oriented to successful PRRS elimination must be based on accurate, cost-effective, and timely detection of infected animals and herds; and because the muscle transudate ("meat juice") samples have proven to be a useful alternative to serum in epidemiological studies and surveillance for infectious agents. The experiment included in the chapter 5 was focused on evaluated three serological assays to detect PRRSV specific antibodies. We provide guidance and performance estimates for using the PRRSV ELISA and IFAT assays on meat samples. These results will provide support for more reliable surveillance studies based on meat samples collected at slaughter. Further studies in this area should be focused on improve these results and validate them on field conditions.

It is our hope that the research described in this paper provides a framework upon which to build future empirical and theoretical investigations into the biological basis of PRRSV persistence, methods of detection, and effective means of intervention may be undertaken. Studies seeking novel mechanisms and those providing both biological plausibility and epidemiological evidence for existing theories are needed. An understanding of what drives PRRSV persistence may allow better understanding of transmission dynamics, leading to better methods of prevention of annual endemic outbreaks, of pandemics of already existing PRRSV strains, and of novel emerging PRRSV strains.

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